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$GABA_A$ Receptor Subtypes in Spinal Pain Processing

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„Wir streben mehr danach, Schmerz zu vermeiden als Freude zu gewinnen.“

(We will do more to avoid pain than to gain pleasure)

Sigmund Freud (1856-1939)

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I. Summary

The biological importance of the nociception and pain is to alert us of potentially tissue-damaging or noxious events and protect us from damage. However, as a result of injury nerve damage or inflammation, pain can become chronic. It, then, no longer serves a physiological function, but can severly interfere with our well-being. Such chronic pain syndromes are often unresponsive to conventional analgesic treatment and hence constitute a major medical and socio-economical problem worldwide. In the last decade, it has become increasingly clear that a loss of spinal inhibition, normally provided by γ-aminobutyric acid (GABA)ergic and glycinergic interneurons, plays a role in the generation and maintenance of chronic pain. Pharmacological facilitation of GABAergic inhibition could thus be a rational approach to the treatment of chronic pain, but wide-spread sedative action and other undesired actions prevent their use in chronic pain patients.

In the first project of this thesis, $GABA_A$ receptor point-mutated (knock-in) mice have been used, in which specific $GABA_A$ receptor subtypes have been rendered insensitive to diazepam (a classical benzodiazepine). Experiments with these mice demonstrate that pronounced analgesia can be achieved by specifically targeting spinal $GABA_A$ receptors containing the α2 and/or α3 subunits. In line with these findings from genetically modified mice, pharmacological experiments demonstrate that the selective targeting of α2 and/or α3 $GABA_A$ receptors by the non-sedative ('α1-sparing') benzodiazepine-site ligand L-838,417 is highly effective against inflammatory and neuropathic pain, yet devoid of unwanted sedation, motor impairment and tolerance development.

The second project adresses the mechanisms of this analgesia. At the level of the spinal cord, $GABA_A$ receptors are expressed not only postsynaptically on dorsal horn neurons, but are also found at the spinal terminals of primary afferent nociceptors where they contribute to presynaptic inhibition through primary afferent depolarization (PAD). Many of these presynaptic $GABA_A$ receptors belong to the α2 subunit containing type (α2-$GABA_A$ receptors), which mediates most of the analgesic action of spinal diazepam. Experiments with conditional, nociceptor-specific α2-$GABA_A$ receptor deficient (*sns*-$\alpha2^{-/-}$) mice, and conditional (*sns*-$\alpha2^{R/-}$), point-mutated mice, whose primary nociceptor α2-$GABA_A$ receptor subunits have been rendered diazepam-insensitive, revealed unchanged nociceptive baseline thresholds and unchanged inflammatory and neuropathic hyperalgesia, but decreased diazepam-induced analgesia against inflammatory pain.

In the last project, the generation of a novel *Homeobox-8-cre* (*Hoxb8-cre)* transgenic mouse line is reported, which expresses the *cre* recombinase under the transcriptional control of the *Hoxb8* gene. This mouse line shows a *cre* expression pattern monitored by reporter gene

mouse lines (e.g. Rosa26lacZ) suitable for brain-sparing gene deletion experiments In the context of GABAergic analgesia, it will help defining the contritution of spinal versus supraspinal sites to $GABA_A$ receptor-mediated analgesia.

The results from this dissertation contribute to the development of a rational basis for the development of subtype-selective $GABA_A$ receptor ligands for the treatment of chronic pain, which is often refractory to classical analgesics.

II. Zusammenfassung (German Summary)

Schmerz ist eine komplexe Sinneswahrnehmung, die als akutes Geschehen den Charakter eines Warnsignals aufweist. Infolge von Nervenschädigungen oder Entzündungen kann Schmerz jedoch chronisch werden und die ursprüngliche physiologische Funktion verlieren. Chronische Schmerzen führen zu teilweise erheblichen Behinderungen, die nicht nur individuelles Leiden auslösen und die Lebensqualität einschränken, sondern einen Einfluss auf viele Bereiche des Lebens haben. Chronische Schmerzen können nur selten adäquat mit den herkömmlichen Schmerzmitteln (Analgetika) behandelt werden.

In den letzten zehn Jahren wurde dank medizinischer Grundlagenforschung klar, dass ein Verlust an spinaler Hemmung, normalerweise durch GABAerge und glycinerge Interneuronen vermittelt, eine wesentliche Rolle in der Entstehung und Verlauf des chronischen Schmerzes spielt. Eine pharmakologische Verstärkung der GABAergen Hemmung sollte daher einen rationalen Ansatz zur Behandlung von chronischen Schmerzen bieten. Eine generelle Verstärkung der GABAergen Hemmung führt jedoch nicht selten auch zu unerwünschten Wirkungen (z.B. Sedation), die den Einsatz solcher Medikamente in der Klinik stark einschränken.

Im ersten Projekt dieser Dissertation wurden genetisch manipulierte Mäusen untersucht, die Punktmutationen an verschiedenen $GABA_A$ Rezeptor Untereinheiten aufweisen, welche zur Insensitivität gegenüber klassischen Benzodiazepinen (z.B. Diazepam) führen. Experimente mit diesen Mäusen zeigten, dass eine gezielte Aktivierung von spinalen $GABA_A$ Rezeptoren, die die $\alpha 2$ und/oder $\alpha 3$ Untereinheiten haben, zur Analgesie führen. Diese Ergebnisse konnten pharmakologisch untermauert werden, indem ein subtypspezifischer (keine pharmakologische $\alpha 1$ Aktivität), nicht-sedativer Ligand (L-838,417) an der Benzodiazepin-Bindungsstelle, verwendet wurde. Dieser Ligand führte in Entzüngsschmerz- und Neuropathiemodellen zur Analgesie ohne Sedation, motorische Störungen oder Gewöhnungseffekte aufzuweisen.

Das zweite Projekt ging dem Mechanismus dieser spinalen Analgesie nach. Auf der Ebene des Rückenmarks sind $GABA_A$ Rezeptoren nicht nur postsynaptisch auf Neuronen im Vorderhorn des Rückenmarks präsent, sondern auch auf den spinalen Endigungen von nociceptiven Afferenzen, wo sie zur präsynaptischen Inhibition durch das Phänomen der primär afferenten Depolarisattion (PAD) beitragen. Viele dieser präsynaptischen $GABA_A$ Rezeptoren enthalten die $\alpha 2$ Untereinheit, welche sich im ersten Projekt als wichtigste Untereinheit für die spinale analgetische Diazepam-vermittelte Wirkung erwiesen hatte.

Experimente mit Mäusen, die eine Nociceptor-spezifische Deletion des $GABA_A$ Rezeptor $\alpha 2$ aufweisen (sns-$\alpha 2^{-/-}$ Mäuse), oder eine Diazepam-insensitive $\alpha 2$ Untereinheit exprimieren

(sns-$\alpha 2^{R/-}$ Mäuse), zeigten, dass sowohl die basale Schmerzschwelle als auch der Verlauf der Hyperalgesie in Entzündungs- und Neuropathiemodellen in diesen Mäusen nicht vermindert waren. Weitere Experimente konnten jedoch zeigen, dass im Entzündungsmodell die Diazepam-induzierte Analgesie deutlich vermindert war.

Im letzten Projekt ist die Entwicklung eine neuen transgenen Mauslinie (*Hoxb8-cre*) beschrieben, welche die *Cre* Recombinase unter der transkriptionalen Kontrolle des Entwicklungsgens *Hob8* exprimiert. Die morphologische Charakterisierung dieser Mauslinie mittels Reporter-Mauslinien (z.B. Rosa26lacZ) zeigte, dass diese Mauslinie geeignet ist, um Gene im Nervensystem unter Aussparung des Gehirn zu deletieren. Im Kontext der GABAergen Analgesie könnte diese Mauslinie daher zur weiteren Klärung der GABAergen Analgesie der spinalen versus supraspinaler Ebene dienen.

Die Ergebnisse dieser Dissertation haben zum Verständnis und zur Grundlage eines rationalen Ansatzes für die Entwicklung von Subtyp-spezifischen $GABA_A$ Rezeptor Liganden beigetragen, welche eventuell zur Behandlung von chronischen Schmerzen eingesetzt werden können.

INTRODUCTION

Parts of this section (chapter 1.2.3) have previously been published as a book chapter:

Zeilhofer HU, **Witschi R**, Johansson T: **Fast Inhibitory Transmission of Pain in the Spinal Cord**" in: Malcangio M: Synaptic Plasticity in Pain. Springer, New York, 2009.

1. General Introduction

1.1 Definition and Epidemiology of Pain

Pain is defined by the International Association for the Study of Pain (IASP) as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Bonica, 1979). A recent telphone survey in Europe found that around 20% of individuals have some form of chronic pain defined in this study as pain lasting for at least 6 months (Breivik et al., 2006). Chronic pain hence constitutes a major medical and socio-economical problem. According to the American Chronic Pain Association (www.theacpa.org), the annual total of both direct and indirect costs of chronic pain are estimated to be as high as $294.5 billion per year, with back pain alone accounting for more than $100 billion per year.

Plenty of evidence indicates that chronic pain is not just prolonged acute pain, but results at least in parts from plastic changes in the processing of sensory and nociceptive (painful) stimuli at all levels of the neuraxis. Much has been learned about molecular and cellular processes underlying the transduction and transmission of acute nociceptiove stimuli, but our knowledge about the pathology underlying chronic pain is still very limited. A similar situation exists in pain treatment. Acute pain can often be controlled effectively with the available analgesics, in particular with opioids, such as morphine, and non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the formation of pronociceptive prostaglandins through blockade of the cyclooxygenases, COX-1 and COX-2. In chronic pain syndomes, however, reduced tolerability and efficacy often limit the therapeutic effects of currently available analgesics (Pezet and McMahon, 2006). Chronic use of opioids can lead to physical dependence and addiction, while conventional NSAIDs can cause severe gastrointestinal side effects and both conventional NSAIDs and COX-2-selective agents increase the risk of adverse cardiovascular events. Furthermore, not all pain forms respond well to opioids or NSAIDs, and many forms of chronic pain are largely resistant to these analgesics. Because of these limitations, many efforts are currently undertaken to better understand the neurobiological bases of chronic pain and to foster the development of novel therapeutic approaches.

1.2 Neurobiology of Pain

The next chapters shall provide an overview over the neuroanatomical and physiological substrates of acute nociception and introduce concepts of spinal mechanisms of peripheral and central sensitization.

1.2.1 Neuroanatomical and Physiological Substrates of Nociception

All higher organisms process specialized nerve cells, called nociceptors, which consitute a class of primary sensory neurons dedicated to the detection of noxious (potentially tissue damaging) stimuli. The peripheral axons of these noccieptors innervate the skin, many visceral organs, and skeletal and cardiac muscle where they are often found in association with blood vessels. The cell bodies of nociceptors innervating the head are located in the trigeminal ganglia, while the others are located in the dorsal root ganglia (DRG). The central axons of nociceptors terminate in the superficial layers of ipsilateral trigeminal nucleus or in spinal dorsal horn, respectively. Most nociceptive nerve fibers are thin slowly conducting nerve fibers and belong either to the unmyeliated C fiber or to the thinly myelinated Aδ fiber class. However, some nociceptors are also found among the think myelinated Aβ fibers. The majority of the touch-sensitive (low-threshold) mechanoreceptors belong to this fiber class. They have the fastest conduction velocities of all sensory nerve fibers and before projecting to directly to the brainstem they send collaterals to neurons in the deeper laminae of the spinal cord dorsal horn.

Different nociceptor types trigger different pain sensations. Activation of Aδ fibers results in a fast sharp and clearly localized pain, while C fiber activation evokes a more prolonged dull and burning pain sensation. There is also evidence that different types of nociceptors are activated by different stimuli. At least in mice, acute noxious mechanical stimuli are mainly detected by Aδ fibers and non-peptidergic (isolectin B4-positive) C fibers, while heat responses are triggered by activation of peptidergic C fibers (Basbaum et al., 2009; Seal et al., 2009). The latter class of nociceptors contains in addition to the fast excitatory neurotransmitter L-glutamate, neuropeptides in particular calcitonin gene-related peptide (CGRP), which upon release in the peripheral tissue acts as a vasodilator and mediates the so called flare response after C fiber stimulation. Some mechano-insensitive (“silent”) nociceptors aquire mechanosensitivity after prolonged stimulation or under inflammatatory conditions (Schmidt et al., 2000; Schmidt et al., 2002).

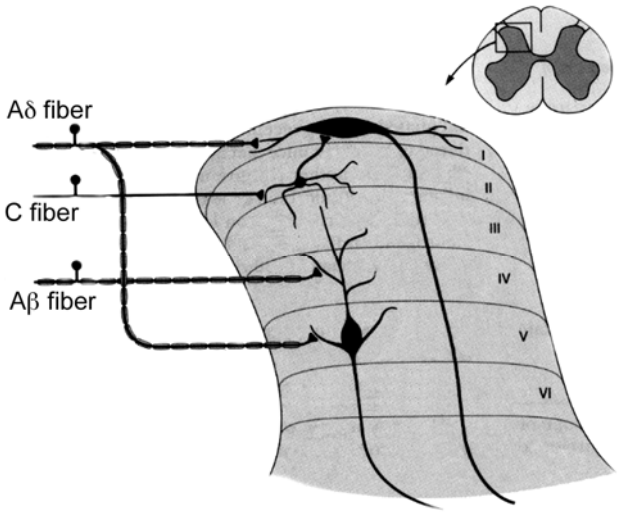

Figure 1. **Spinal Cord Dorsal Horn.**

Nociceptive afferent fibers terminate in the superficial layers of the dorsal horn of the spinal cord. Projection neurons in lamina I receive direct input from Aδ and C nociceptors. Lamina V neurons are predominantly of the wide dynamic-range type. They receive input from low-threshold large-diameter myelinated fibers (Aβ) of mechanoreceptors as well as both direct and indirect input from nociceptive afferent fibers (Aδ and C). In this figure the lamina V neuron sends a dendrite up through lamina IV, where it is contacted by the terminal of an Aβ primary afferent. A dendrite in lamina III arising from a cell in lamina V is contacted by the axon terminal of a lamina II interneuron. Adapted from (Kandel et al., 2000).

In the last 15 years, researchers have begun to understand the signal transduction in nociceptor terminals on a molecular level. It has been found that transient receptor potential (TRP) channels play a crucial role in the transduction of thermal (heat and cold) stimuli. One of these TRP channels, the TRPV1 (Davis et al., 2000; Prescott and Julius, 2003; Eckert et al., 2006) channel, serves as an integrator of different noxious stimuli including heat (>43°C) and tissue acidosis in inflammation and ischemia. This ion channel can be specifically activated by capsaicin, the hot tasting ingredient of peppers. TRPA1 is another TRP channel expressed by nociceptors, which is activated by a wide variety of chemical stimuli including tear gas, mustard oil, and by noxious cold. These TRP channels are non-specific cation channels, which upon opening depolarize the nociceptor terminal to levels sufficient to elicit action potentials.

The central axons of Aδ and C nociceptors terminate mainly in the superficial layers (laminae I and II, (Rexed, 1952)) of the spinal cord dorsal horn with peptidergic C fibers terminating mainly in lamina I and II outer (IIo) and non-peptidergic C fibers terminating mainly in lamina II inner (IIi) (Hunt and Mantyh, 2001; Zylka, 2005). Both nociceptor types use L-glutamate as their principle fast exciatory neurotransmitter and excite “pain-specific” projection neurons located in lamina I through either mono- or polysynaptic connections. L-glutamate acts primarily on ionotropic glutamate receptors, of the α-amino-3-hydoxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate type. *N*-methyl-*D*-aspartate (NMDA) receptors

contribute only little to acute postsynaptic excitation because these receptors are tonically blocked by extracellular Mg^{2+} (Davies and Lodge, 1987; Dingledine et al., 1999) but are important for plastic changes in nociceptive processing.

The axons of projection neurons cross the midline and ascend mainly in the spinoreticular and spinothalamic tract to the brainstem and the thalamus. The perception of pain involves sensory-discriminative, affective-motivational and cognitive-evaluative dimensions. These different aspects of pain are represented in distinct pain pathways and brain regions activated during the experience of pain (the so called pain matrix). The ‘‘lateral pain system’’ includes the primary (S1) and secondary (S2) somatosensory cortices that receive input from the lateral thalamic nuclei and reflects the sensory and discriminative components (location and intensity) of pain. Another pathway (‘‘medial pain system“) branches at the level of the medulla and ascends via the medial thalamus to hypothalamic nuclei, limbic regions including the anterior cingulate cortex (ACC), the insula cortex (IC) and onto prefrontal areas, all of which are involved in the control of emotion, arousal and attention. This medial pain pathway is therefore proposed to mediate the unpleasant, affective dimensions of pain, and the motivation to escape from the noxious event (Treede et al., 1999; Price, 2000; Brooks and Tracey, 2005; Bushnell, 2006).

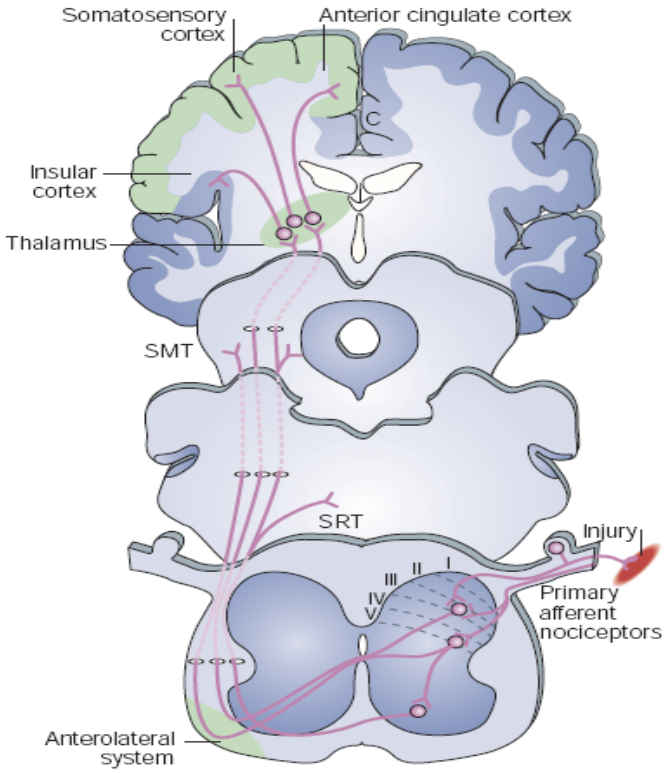

Figure 2. **Schematic of afferent pathways underlying the sensation of pain.**

Injury activates primary afferent nociceptors (PAN), which transmit information to the dorsal horn of the spinal cord. The terminals of the PAN contact neurons in specific laminae of the dorsal horn where they release glutamate and neuro-peptides to activate second order neurons. The axons of nociceptive dorsal horn neurons cross to the contralateral anterolateral quadrant to form an ascending tract, which terminates in the brainstem and several distinct areas of the thalamus, which contain higher order neurons that project to various cortical regions that mediate different aspects of the pain experience. These regions include somatosensory, anterior cingulate and insular cortices. SMT, spinomesencephalic tract; SRT, spinoreticular tract. Adapted from (Fields, 2004).

1.2.2 Peripheral and Spinal Pain Sensitization

Many chronic pain forms are accompanied by abnormal pain sensitivity, which can be classified as hyperalgesia (increased pain to mild noxious stimuli), allodynia (touch-evoked pain) or spontaneous pain ocurring in the absence of any sensory stimulation. Hyperalgesia occurring at the site of injury is called primary hyperalgesia, while enhanced pain sensitivity in uninjured healthy tissue is termed secondary hyperalgesia. Both peripheral and central processes can contribute to primary hyperalgesia and probably also to spontaneous pain, whereas secondary hyperalgesia and allodynia originate exclusively from central sensitization.

Peripheral hyperalgesia is frequently triggered by pro-inflammatory mediators including among others prostaglandins (mainly PGE_2 and PGI_2), bradykinin, and NGF, which increase the excitability of nociceptors. Some of these mediators have been shown to facilitate the activation of TRP channels in particular of TRPV1 channels and of voltage-gated sodium channels (e.g. NaV1.8) through protein kinase A (PKA) or PKC dependent phosphorylation or through changes in the expression of the respective genes (Tate et al., 1998; Michael and Priestley, 1999; Bhave et al., 2002; Hudmon et al., 2008).

Several different concepts exist about the changes in the spinal processing of sensory and nociceptive processing that underlie chronic pain syndromes. Intense and prolonged nociceptive input to the spinal dorsal horn leads to short-term and long-term increases in synaptic excitability of dorsal horn neurons. Repetitive stimulation of C fibers leads to a frequency-dependent temporal summation of postsynaptic potentials which results in increased excitability of dorsal horn neurons (wind-up; (Herrero et al., 2000)). A wide variety of mediators and receptors including glutamate acting on NMDA receptors, substance P on NK1 receptors, probably contribute to this form of short-term plasticity. A behavioral correlate of wind-up can be observed after repeated heat or noxious mechanical stimulation, where felt pain intensities increase with each successive stimulus even though the stimulus intensity does not change (Price et al., 1977; Staud et al., 2003).

Another longer lasting phenomenon of increased excitability is long-term potentiation (LTP). It has first been described in the hippocampus (Bliss and Lomo, 1973), where it is generally believed to be a cellular correlate of learning and memory. LTP does also occur at synapses between C fibers and lamina I spinobrachial projection neurons (Randic et al., 1993; Liu and Sandkuhler, 1995; Sandkuhler and Liu, 1998; Ikeda et al., 2003; Sandkuhler, 2007). In contrast to hippocampal LTP, C fiber-induced LTP in the dorsal horn can be elicited by stimulation frequencies as low as 1-2 Hz, i.e. at frequencies occurring naturally in C fibers during physiological activation (Drdla and Sandkuhler, 2008). A long-lasting increase in the efficacy of synaptic transmission between C fiber nociceptors and spinal projection neurons very likely contributes to enhanced pain sensitivity following intense nociceptive input to the

spinal cord. It can hence explain the long-lasting hyperalgesia observed in chronic pain patients.

However, other symptoms of chronic pain in particular allodynia cannot easily be explained through such processes. Allodynia (or touch-evoked pain) is by definition a form of pain which is triggered by activation of non-nociceptive (low-threshold) Aβ mechanoreceptive fibers. One concept explains allodynia through a suprathreshold activation of normally "pain-specific" projection neurons via polysynaptic connections formed by Aβ fibers and excitatory interneurons. Under healthy conditions, this pathway is silent, but can become active under conditions of reduced GABAergic or glycinergic synaptic inhibition (see also figure 4).

1.2.3 Diminished Synaptic GABAergic and Glycinergic Control

Already in 1965, the gate control theory of pain (Melzack and Wall, 1965) attributed to inhibitory interneurons located in the superficial dorsal horn a critical role in sensory and nociceptive processing, and proposed that these neurons would determine whether nociceptive input coming from the periphery was transmitted through the spinal cord to higher CNS areas where pain becomes conscious.

Histological experiments have later confirmed the existence of different inhibitory interneurons in the spinal dorsal horn. Studies using antisera against GABA and glycine demonstrated the presence of inhibitory interneurons throughout the grey matter of the spinal dorsal horn. In its superficial layers, about 30% of lamina I and II neurons exhibit GABA-like immunoreactivity (Todd and Sullivan, 1990). Many of these GABAergic neurons (33, 43% in lamina I, II, respectively) are also immunoreactive for glycine. This is supported by more recent experiments that employed genetically modified mice expressing enhanced green fluorescent protein (EGFP) under the transcriptional control of the glutamic acid decarboxylase (GAD)67 (*gad1*) gene, a marker gene for GABAergic neurons (Oliva et al., 2000; Tamamaki et al., 2003). Coronal sections of spinal cords from these mice show numerous EGFP labeled neurons in lamina I-III and around the central canal but only few neurons in the deeper dorsal horn. Glycinergic neurons tagged with EGFP expressed under the control of the GlyT2 promotor gene show a somewhat different distribution (Zeilhofer, 2005) with a high abundance of these neurons in the deeper laminae (laminae III-V) and relatively few neurons in laminae I and II.

The inhibitory action of GABA appears to be more complex in the spinal dorsal horn than in most parts of the brain, where GABA inhibits neuronal excitability primarily through hyperpolarization and the activation of a shunting conductance. At the level of the spinal cord, $GABA_A$ receptors are expressed not only postsynaptically on dorsal horn neurons, but are also found at the spinal terminals of primary afferent nerve fibers where they contribute to presynaptic inhibition through primary afferent depolarization (PAD) (Eccles et al., 1961). In

contrast to adult central neurons (including postsynaptic dorsal horn neurons), which are hyperpolarized by GABA, primary sensory neurons including their spinal terminals are usually depolarized due to an unusually high intracellular chloride concentration. This depolarization originates from a very particular expression pattern of chloride transporters with high expression of NKCC1, which transports chloride, sodium and potassium into the cell (Sung et al., 2000; Price et al., 2006), and low expression of KCC2 (Rivera et al., 1999), which transports chloride and potassium out of the cell. Subthreshold primary afferent depolarization (PAD) causes presynaptic inhibition, which reduces transmitter release from primary afferent nerve terminals possibly through inactivation of voltage-gated Ca^{2+} channels, or through activation of a shunting conductance in the intraspinal segment of the incoming axon, which would interfere with the propagation of the incoming action potentials. It is believed that primary afferent depolarizations can become suprathreshold under pathological conditions and would then facilitate nociception (Willis, 1999). Figure 4 summarizes current concepts of the integration of inhibitory GABAergic and glycinergic neurons in the dorsal horn neuronal circuit.

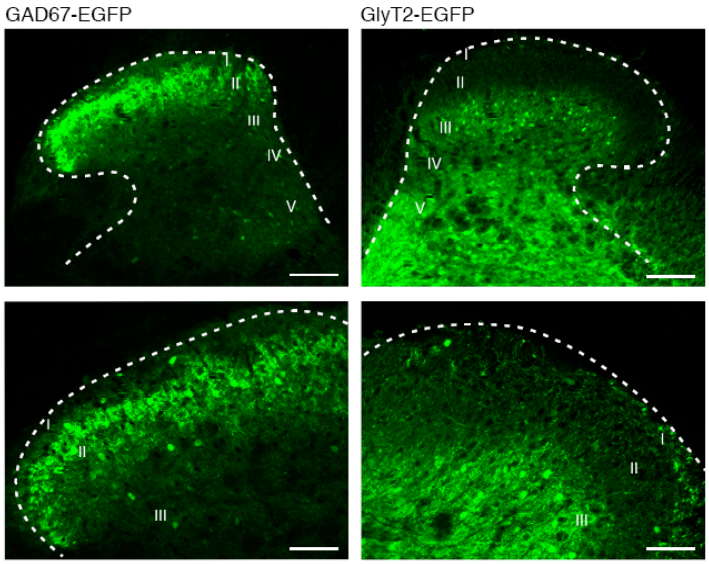

Figure 3. **Distribution of GABAergic and glycinergic neurons.**

Transverse sections of the lumbar spinal cord of GAD67-EGFP (Tamamaki et al., 2003) and GlyT2-EGFP (Zeilhofer, 2005) transgenic mice at different magnification. GAD67-EGFP tagged somata are most prominent in lamina II, whereas GlyT2-EGFP positive somata are most abundant in the deeper dorsal horn (lamina III and V). Scale bars: 100 µm and 50 µm, top and bottom panels, respectively. Adapted from (Zeilhofer et al., 2009).

A critical role of the two inhibitory neurotransmitters GABA and glycine in the spinal control of nociception was confirmed in behavioral experiments when several groups tested the effects of spinally applied bicuculline and strychnine, blockers of $GABA_A$ and glycine receptors, on nociceptive behavior in rodents (Roberts et al., 1986; Yaksh, 1989; Yamamoto and Yaksh,

1993; Sivilotti and Woolf, 1994; Malan et al., 2002). It was found that both antagonists increased nociceptive reactions elicited by exposure to noxious stimuli and induced nociceptive reactions in response to innocuous stimuli such as light touch. The spinal pharmacological antagonism of $GABA_A$ and glycine receptors with bicuculline or strychnine thus induces typical symptoms of chronic pain such as tactile allodynia and hyperalgesia. Conversely, intrathecal application of GABA reversed thermal and mechanical sensitivity in rats with chronic constriction nerve injury (Eaton et al., 1999). Most importantly, it has recently been demonstrated that a loss of inhibitory pain control occurs also endogenously in the natural course of inflammatory and neuropathic pain and after intense nociceptive input up te spinal dorsal horn. Diminished inhibitory pain control at the spinal dorsal horn level may thus constitue a major factor in the generation and maintenance of central pain sensitization.

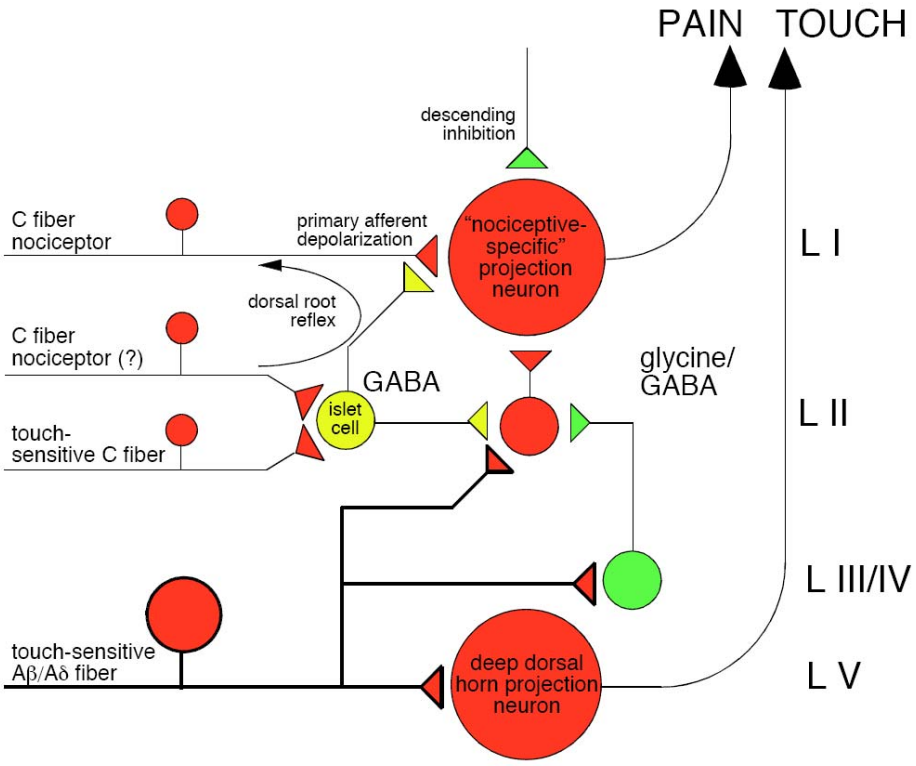

Figure 4. **Possible integration of inhibitory dorsal horn neurons in spinal pain processing circuits.**

GABAergic islet cells (yellow) in the substantia gelatinosa (L II) receive monosynaptic input from C fibers, which are believed to be mainly non-nociceptive touch-sensitive (Bennett et al., 1980). These lamina II GABAergic cells form different synaptic connections, with presynaptic terminals of primary afferent fibers, where they induce primary afferent depolarization, and with intrinsic superficial dorsal horn neurons, where they cause classical postsynaptic inhibition. They probably also synapse to lamina I (L I) projection neurons. Mixed GABA/glycinergic cells (green) and pure glycinergic cells are located mainly in the deeper laminae (L III and V). They are probably excited primarily by mechanosensitive fibers and cause postsynaptic inhibition of excitatory interneurons and possibly also of projection neurons. Removal of this inhibition leads to polysynaptic excitation of normally nociceptive specific neurons in lamina I and induces touch-evoked pain (allodynia). Excitatory neurons and terminals (red). Adapted from (Zeilhofer et al., 2009).

Synaptic Dis-inhibition in Inflammatory Pain

Peripheral inflammation induces a pronounced increase in the spinal production of prostaglandin E2 (PGE_2), a key mediator of central inflammatory hyperalgesia. The two enzymes required for inflammation-induced PGE_2 production, cyclooxygenase-2 (COX-2) and inducible microsomal prostaglandin E synthase 1 (mPGES-1), are up-regulated in the dorsal horn within hours after induction of peripheral inflammation (Beiche et al., 1996; Murakami et al., 2000; Samad et al., 2001; Claveau et al., 2003). A major down-stream effect of spinally produced PGE_2 is the reduction of glycinergic transmission in the superficial dorsal horn (Ahmadi et al., 2002). This inhibition occurs through a postsynaptic mechanism involving the activation of PGE_2 receptors of the EP2 subtype, subsequent cAMP production and activation of protein kinase A (PKA). Activated PKA phosphorylates and inhibits a specific isoform of gylcine receptors containing the $\alpha 3$ subunit, which in the spinal cord is distinctly expressed in the superficial layers (Harvey et al., 2004). Interestingly, PGE_2 mediated inhibition of glycine receptors occurs in the majority of excitatory superficial dorsal horn neurons (Ahmadi et al., 2002; Reinold et al., 2005), in a pattern which is reminiscent of the PKCγ activation seen in vivo after blockade of glycine receptors with strychnine (Miraucourt et al., 2007). Work in EP2-receptor-deficient mice and in mice lacking the glycine receptor $\alpha 3$ (Gly$R\alpha 3$) subunit has shown that the pro-nociceptive actions of PGE_2 are virtually absent in these mice and that inflammatory pain is strongly reduced (Harvey et al., 2004; Reinold et al., 2005). Interestingly, the development of neuropathic pain in chronic constriction injury (CCI) model is not altered in EP2 or Gly$R\alpha 3$ deficient mice as compared to wild type mice (Hosl et al., 2006). A similar pattern has been reported earlier in mice carrying a null mutation in the regulatory subunit of neuronal protein kinase A (Malmberg et al., 1997). These mice also showed reduced nociceptive responses to intrathecal PGE_2, but exhibited normal pain responses in the CCI model.

It should be noted that inflammation can also up-regulate GABAergic inhibition in the spinal dorsal horn through production of endogenous $3\alpha 5\alpha$-neurosteroids (Inquimbert et al., 2007). These neurosteroids are positive allosteric modulators of $GABA_A$ receptors (Hosie et al., 2006), which, at least in the spinal dorsal horn, target primarily extrasynaptic $GABA_A$ receptors (Mitchell et al., 2007). They are regularly produced in the spinal cord during development, but are normally absent in the adult (Keller et al., 2004). In response to a peripheral inflammatory stimulus, they re-appear in the spinal cord and may limit inflammatory thermal hyperalgesia (Poisbeau et al., 2005; Vergnano et al., 2007).

Synaptic Dis-inhibition in Neuropathic Pain

At least two mechanisms have been proposed that would cause synaptic dis-inhibition after peripheral nerve injury. The group of Clifford Woolf proposed that GABAergic synaptic transmission was diminished in spinal cord slices of neuropathic rats (Moore et al., 2002) This loss was accompanied by a reduction in GAD65 immunoreactivity in the dorsal horn ipsilateral to the peripheral nerve injury. Whether or not this loss is due to an apoptotic death of GABAergic interneurons, as suggest by the authors, is still controversial (Polgar et al., 2005; Scholz et al., 2005; Polgar and Todd, 2008). Since GAD antisera mainly stain presynaptic boutons (Mackie et al., 2003), it is conceivable that only GABAergic boutons degenerate, while their neuronal somata remain intact.

Another pathway leading to diminished synaptic inhibition depends on the activation of spinal micoglia, which has been recognized as a key event in the generation of neuropathic pain following peripheral nerve damage (Tsuda et al., 2003; Scholz and Woolf, 2007). A possible link between microglia activation and altered neuronal processing of sensory information is the release of brain-derived neurotrophic factor (BDNF) from microglia cells and the subsequent trkB-mediated down-regulation of the potassium chloride co-transporter KCC2 in dorsal horn neurons (Coull et al., 2003; Coull et al., 2005). KCC-2 is required for the maintenance of a low intracellular chloride concentration (Rivera et al., 1999) and reduced expression of KCC2 increases intracellular chloride concentrations and renders GABAergic and glycinergic input less inhibitory, or may even turn it into excitation.

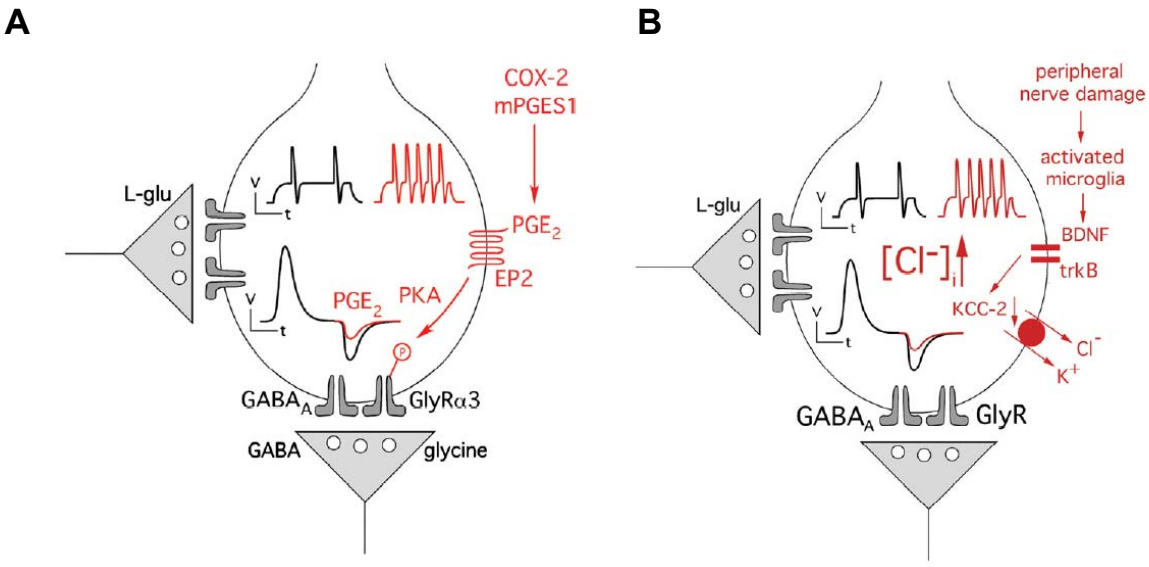

Figure 5. **Diminished synaptic GABAergic and glycinergic control.**

(A) Dis-inhibition in inflammatory pain states. Cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase (mPGES1) become induced in the spinal cord in response to inflammation in peripheral tissues and produce PGE_2. PGE_2 binds to PGE_2 receptors of the EP2 subtype, which increase cAMP levels and activate protein kinase A (PKA). PKA then phosphorylates and inhibits a specific subtype of glycine receptors containing the $\alpha 3$ subunit, which normally control the excitability of superficial dorsal horn neurons. This dis-inhibition facilitates the firing of these neurons and promotes transmission of nociceptive signals through the spinal cord to higher brain areas where pain becomes conscious. (B) Dis-inhibition in neuropathic pain states. Spinal microglia activated in response to peripheral nerve damage, releases brain-derived neurotrophic factor (BDNF). BDNF subsequently down-regulates the potassium-chloride cotransporter (KCC-2), which normally keeps intracellular chloride concentration ($[Cl^-]_i$) low. This down-regulation reduces the inhibitory action of $GABA_A$ and glycine receptors. In some neurons GABAergic and glycinergic inhibition may become even depolarizing and excitatory. This dis-inhibition promotes the firing of dorsal horn neurons and the transmission of nociceptive signals. Adapted from (Zeilhofer and Zeilhofer, 2008).

Synaptic Dis-inhibition in Activity-Dependent Sensitization

A loss of dorsal horn synaptic inhibition can also occur in the absence of inflammation and nerve injury. Selective activation of C fiber nociceptors with capsaicin induces mechanical and thermal hyperalgesia at the site of injection (primary hyperalgesia) and in addition an exclusively mechanical sensitization (pin-prick hyperalgesia and touch-evoked pain) in a surrounding healthy skin area (Treede and Magerl, 2000). Plenty of evidence suggests that this secondary hyperalgesia is of central (spinal) origin (Woolf, 1983), and involves diminished synaptic inhibition (Sivilotti and Woolf, 1994). It has recently been demonstrated by our goup (Pernia-Andrade et al., 2009) that intense C fiber input reduces the release of GABA and glyine from inhibitory dorsal horn neurons through the production of endocannabinoids and the subsequent activation of CB1 cannabinoid receptors on inhibitory axon terminals.

1.3 GABA Receptors

GABA receptors are activated by the neurotransmitter GABA, the major inhibitory neurotransmitter in the vertebrate central nervous system. $GABA_A$ receptors are ligand-gated ion channels (also known as ionotropic receptors) permeable to chloride and bicarbonate, whereas $GABA_B$ receptors are G protein-coupled or metabotropic receptors. $GABA_A$ receptors inhibit neuronal activation through hyperpolarization and the activation of a shunting conductance. $GABA_B$ receptors were originally distinguished from $GABA_A$ receptors on the basis of their pharmacological properties (Bowery et al., 1980). They reduce neuronal excitability through the opening of inwardly rectifying K^+ channels and the inhibition of voltage-gated calcium channels (VGCCs). They also inhibit adenylyl cyclases (Pan et al., 2008). A subclass of ionotropic $GABA_A$ receptors, insensitive to bicuculline and exclusively composed of ρ subunits (Shimada et al., 1992; Kusama et al., 1993b; Kusama et al., 1993a), has originally been designated as $GABA_C$ receptors but is now termed $GABA_{A\text{-}\rho}$ (Olsen and Sieghart, 2008).

1.3.1 $GABA_A$ Receptors

$GABA_A$ receptors are members of the Cys-loop ligand-gated ion channel superfamily (Mohler, 2006), which includes nicotinic acetylcholine receptors, $GABA_A$ receptors, glycine and $5\text{-}HT_3$ receptors. This ion channel family is characterized by the presence of an extracellular loop formed by a disulfide bond between two cysteine residues. $GABA_A$ receptors are heteropentameric ion channels assembled from a repertoir of at least 19 subunits ($\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, δ, ε, θ, π, $\rho_{1\text{-}3}$). The most common subtype in the CNS is composed of two α, two β, and one γ subunit (Mohler, 2006). The receptor is activated upon binding of two GABA molecules, at an interface formed by an α and a β subunit for each molecule (Mohler, 2006). Activation of $GABA_A$ receptors by GABA and other agonists (e.g. muscimol) leads to an increase in chloride conductance driving the membrane potential towards the chloride equilibrium potential which is about -65 mV in most adult central neurons, thereby decreasing the excitability of the neuron. There are also reports of excitatory effects of $GABA_A$ receptor activation, which occur as a consequence of increased intracellular cloride concentrations present e.g. during development (Ben-Ari et al., 1997; Taketo and Yoshioka, 2000) or in certain cell populations, such as some hippocampal interneurons (Lamsa and Taira, 2003) or, as discussed above, in DRG neurons (see 1.2.3).

$GABA_A$ receptors contain several allosteric binding sites, which modulate their activity. These allosteric sites are the targets of various drugs, including benzodiazepines, barbiturates, ethanol, neuroactive steroids, inhaled anaesthetics, and others (Mohler, 2006). $GABA_A$ receptors containing the α1, α2, α3 or α5 subunit associated with a γ2 subunit are

benzodiazepine-sensitive (Wieland et al., 1992). Once benzodiazepines have bound, they lock the $GABA_A$ receptor into a conformation that increases the affinity for GABA, and the frequency of channel openings. Benzodiazepines also potentiate the effect of muscimol, another $GABA_A$ receptor agonist, on $GABA_A$ receptors (Akhondzadeh and Stone, 1998; Akhondzadeh et al., 2002). A minority of $GABA_A$ receptors contains instead α4, α6, or ρ-subunits and is insensitive to classical benzodiazepines (1*H*-Benzo-1,4-diazepine). Some of these receptors are however subject to modulation by other drugs such as neurosteroids (Lambert et al., 2003; Belelli and Lambert, 2005; Belelli et al., 2006) and ethanol (Kumar et al., 2009).

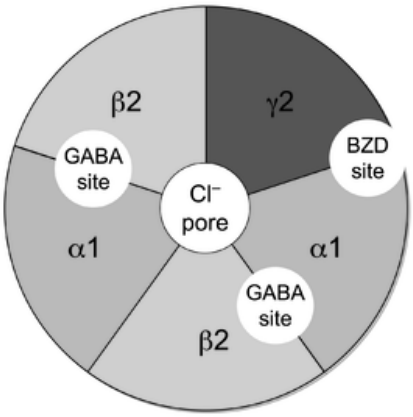

Figure 6. **Benzodiazepine-sensitive $GABA_A$ receptor.**

Schematic illustration of a $GABA_A$ receptor protein ($(\alpha1)_2(\beta2)_2(\gamma2)$) which illustrates the five combined subunits that form the protein, the chloride (Cl^-) ion channel pore, the two GABA active binding sites at the α1 and β2 interfaces, and the benzodiazepine (BDZ) allosteric binding-site at the α1 and γ2 interface.

1.4 Analysis of $GABA_A$ Receptor Functions Using Genetically Modified Mice

In the last decade, many efforts have been made to study the functions of specific $GABA_A$ receptor isoforms *in vivo* with knock-out mice which lack different $GABA_A$ receptor subunits. In many of these knock-out mice, compensatory up- or down-regulations of other $GABA_A$ receptor subunits (α1 knock-out, (Sur et al., 2001; Vicini et al., 2001) limit the interpretation of these studies. Other knock-out lines such as the γ2 knock-out (Gunther et al., 1995), exhibited lethal phenotypes. In contrast, the β2 knock-out had no obvious behavioral consequences (Sur et al., 2001) although the β2 subunit is the most abundant of all β subunits.

Important insights into the understanding of the contribution of $GABA_A$ receptor isoforms to the different *in vivo* actions of benzodiazepines came from the generation of $GABA_A$ receptor

point-mutated ("knock-in") mice, which carry $GABA_A$ receptor α subunits rendered benzodiazepine-insensitive through the exchange of a single amino acid (Rudolph et al., 2001). Knock-in mice containing these point-mutations (α1(H101R), α2(H101R), α3(H126R), α5(H105R) (Rudolph et al., 1999; Low et al., 2000; Crestani et al., 2001; Crestani et al., 2002) were generated to characterize the involment of defined $GABA_A$ receptor benzodiazepine-sensitive α subtypes in different actions of diazepam. Most importantly, it has been found that diazepam-induced sedation and anterograde amnesia are mediated through α1-containg $GABA_A$ receptors (Rudolph et al., 1999; McKernan et al., 2000), while the α2 subunit is required for the anxiolytic (Low et al., 2000) and myorelaxing effects (Crestani et al., 2001) of benzodiazepines. α5 subunits seem to be involved in learning and memory (Crestani et al., 2002). With the exception of the α5(H105R) mice, the three other α subunit knock-in mouse lines did not show a decrease in the mutated subunit or apparent compensatory up- or down-regulation of other $GABA_A$ receptor subunits.

1.5 Conditional Gene Deletion Using the *cre/loxP* System

The global knock-out of certain genes can lead to embryonic lethality, thus making it impossible to obtain adult animals for the analysis of the gene function *in vivo*. The *cre/loxP* recombination provides a special type of site-specific gene recombination (Sauer et al., 1987; Sauer and Henderson, 1988a, 1988b). *creloxP* recombination involves the targeting of a specific sequence of DNA and its splicing with the help of an enzyme called *cre* recombinase. The *cre* recombinase is a 38-kDa protein that recognizes a 34-bp DNA segment termed "locus of crossing-over of P1" (*loxP*). It has first been discovered in the bacteriophage P1 (Orban et al., 1992; Lakso et al., 1996). This minimal target sequence site is unlikely to occur randomly in the mouse genome and is small enough to be "neutral" when integrated into chromosomal DNA. If two *loxP* sites are located on the same DNA molecule, *cre* causes inversion or excision of the intervening DNA segment depending on their respective orientation. Two transgenic mouse lines are required to generate tissue-specific gene deletions. The first mouse line expresses the *cre* recombinase under the control of a tissue-specific promoter. The second carries *loxP* sites around the gene (essential exon) of interest ("floxed gene"). In co-transgenic offspring, the gene (or exons) of interest will be removed selectively from cells expressing *cre* recombinase. The *cre* recombinase can also be expressed in a temporally controlled manner. Cre^{ERT2} encodes a *cre* recombinase fused to a mutant estrogen ligand-binding domain (ERT2) that requires the presence of tamoxifen for activity.

EXPERIMENTAL SECTION

2. Aims

As outlined above, several lines of evidence suggest that pathological pain of inflammatory or neuropathic origin converge onto a loss of synaptic inhibition in the spinal dorsal horn. A potentiation of inhibitory neurotransmission should in principle allow compensating for this loss. Benzodiazepines, which facilitate the action of GABA at $GABA_A$ receptors exert clear analgesic or antihyperalgesic actions after local spinal application, both in animal models of pain (Clavier et al., 1992; Sumida et al., 1995) and in patients (Tucker et al., 2004a; Tucker et al., 2004b), for a comprehensive review see (Jasmin et al., 2004). This thesis uses an integrative approach combining pharmacological, behavioral, electrophysiological and morphological experiments in wild type and genetically modified mice to unravel the identity and the localization of the $GABA_A$ receptor subtypes mediating this analgesia.

2.1 Identification of $GABA_A$ Receptor Isoforms Mediating Benzodiazepine-Induced Spinal Analgesia

This aim was achieved with behavioral tests in wild type and $GABA_A$ receptor mutant mice carrying point mutated (diazepam-insensitive) α subunits ($\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$) (Rudolph et al., 1999; Low et al., 2000; Crestani et al., 2001; Crestani et al., 2002). The effect of intrathecally applied diazepam was tested in models of inflammatory, neuropathic and chemically-induced pain. The results from these mutant mouse studies were complemented with experiments employing subtype-specific benzodiazepine binding-site ligands.

2.2 Identification of the Contribution of Presynaptic $GABA_A$ Receptors Located on the Central Terminals of Primary Nociceptive Afferents to the Spinal Control of Nociception

At the spinal cord level, $GABA_A$ receptors can in principle modulate nociceptive processing via at least two sites. Postsynaptically located $GABA_A$ receptors directly reduce the excitability of central dorsal horn neurons, while $GABA_A$ receptors located presynaptically on the spinal terminals of primary afferent nociceptors cause presynaptic inhibition. Morphological data employing immunofluorescence and *in-situ* hybridization have suggested that $\alpha2$-$GABA_A$ receptors, which mediate most of the analgesic effects of spinal benzodiazepines (Knabl et al., 2008), are the major if not the only $GABA_A$ receptor isoform in dorsal root ganglion neurons (Ma et al., 1993). To address the contribution of these

presynaptic $GABA_A$ receptors to the spinal control of nociception and to the analgesic effects of spinal benzodiazepines, mice were generated which carry targeted deletions/mutations of the $GABA_A$ receptor $\alpha 2$ subunit in primary nociceptive neurons. Behavioral experiments were done to analyze the phenotype of $GABA_A$ receptor mutant mice in different models of inflammatory and neuropathic pain in absence of treatment and after intrathecal injection of diazepam.

2.3 Generation of a *cre* Transgenic Mouse Line for Brain-Sparing Conditional Gene Deletion

This project part aimed at the generation of a *cre* transgenic mouse for brain-sparing gene deletion, which would allow to determine the contribution of spinal versus supraspinal $GABA_A$ receptors to benzodiazepine-induced analgesia. To address this aim, it was planned to express the *cre* recombinase under the transcriptional control of the homeobox gene *Hoxb8*, which is expressed during development throughout the spinal cord up to cervical segment C4. Proper expression of the transgene should be verified in morphological experiments performed on tissue from co-transgenic offspring of *cre* transgenic mice crossed with different reporter strains (Rosa26lacZ and RA/EG).

3. Results

3.1 Reversal of Pathological Pain through Specific Spinal $GABA_A$ Receptor Subtypes*

* Knabl J, **Witschi R**, Hösl K, Reinold H, Zeilhofer U, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Möhler H, Zeilhofer HU (2008): Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes. ***Nature*** 451, 330-334.

Abstract

Inflammatory diseases and neuropathic insults are frequently accompanied by severe and debilitating pain, which can become chronic and often unresponsive to conventional analgesic treatment[1,2]. A loss of synaptic inhibition in the spinal dorsal horn is considered to contribute significantly to this pain pathology[3–7]. Facilitation of spinal γ-aminobutyric acid (GABA)ergic neurotransmission through modulation of $GABA_A$ receptors should be able to compensate for this loss[8,9]. With the use of $GABA_A$ receptor point-mutated knock-in mice in which specific $GABA_A$ receptor subtypes have been selectively rendered insensitive to benzodiazepine-site ligands[10–12], we show here that pronounced analgesia can be achieved by specifically targeting spinal $GABA_A$ receptors containing the α2 and/or α3 subunits. We show that their selective activation by the non-sedative ('α1-sparing') benzodiazepine-site ligand L-838,417 (ref. 13) is highly effective against inflammatory and neuropathic pain yet devoid of unwanted sedation, motor impairment and tolerance development. L-838,417 not only diminished the nociceptive input to the brain but also reduced the activity of brain areas related to the associative-emotional components of pain, as shown by functional magnetic resonance imaging in rats. These results provide a rational basis for the development of subtype-selective GABAergic drugs for the treatment of chronic pain, which is often refractory to classical analgesics.

Introduction

More than 40 years ago, the gate control theory of pain[14] proposed that inhibitory neurons in the superficial dorsal horn of the spinal cord control the relay of nociceptive signals (that is, those evoked by painful stimuli) from the periphery to higher areas of the central nervous system. The pivotal role of inhibitory GABAergic and glycinergic neurons in this process has recently been demonstrated in several reports indicating that a loss of inhibitory neurotransmission underlies several forms of chronic pain[3–7]. Despite this knowledge, inhibitory neurotransmitter receptors have rarely been considered as targets for analgesic treatment. In fact, classical benzodiazepines, which are routinely used for their sedative, anxiolytic and anticonvulsant activity, largely lack clear analgesic efficacy in humans when given systemically[15]. To address this obvious discrepancy we investigated the molecular basis of GABAergic pain control in the spinal cord in an integrative approach based on an electrophysiological and behavioural analysis of genetically modified mice and on functional imaging in rats.

Methods

Mice and rats. Behavioural experiments were performed in male and female 7–12-week-old mice or in male 7–12-week-old Wistar rats. Wild-type mice and $GABA_A$ receptor mutant mice (α1(H101R), α2(H101R), α3(H126R) and α5(H105R))[10–12] were maintained on a 129X1/SvJ background. In all behavioural tests, the observer was blinded to the genotype or to the drug treatment. Permission for the animal experiments was obtained from the Regierung von Mittelfranken (ref. no. 612-2531.31-17/03) and from the Veterinäramt des Kantons Zürich (ref. no. 121/2006 and 34/2007).

Drugs. For intrathecal injection in mice, diazepam was dissolved in 10% dimethyl sulphoxide (DMSO), 90% artificial cerebrospinal fluid (ACSF) (vehicle). Total intrathecal injection volume was 5 µl (for details of the injection procedure see ref. 31). Up to a concentration of 20%, intrathecal DMSO had no effect on pain behaviour in mice. For i.p. injection, diazepam was dissolved in 0.3% Tween 80, 99.7% ACSF. Morphine was dissolved in ACSF. L-838,417 synthesized by Anawa was suspended in 0.5% methylcellulose and 0.9% NaCl and was applied to rats either orally or i.p. in a total volume of 200 µl. Flumazenil (10 $mg \cdot kg^{-1}$) and naloxone (10 $mg \cdot kg^{-1}$) were dissolved in DMSO (1%) and injected i.p. in a total volume of 200 µl.

Formalin test. Formalin (5%, 20 µl) was injected subcutaneously into the dorsal surface of the left hindpaw[32]. Flinches of the injected paw were counted for 60 min starting immediately

after formalin injection. Intrathecal drugs (diazepam or vehicle) were injected 10 min before formalin injection. Flumazenil (10 $mg \cdot kg^{-1}$) was injected i.p. 30 min before formalin injection.

Inflammatory pain. Inflammatory pain was assessed in the zymosan A model[33]. In mice, 0.06 mg of zymosan A suspended in 20 µl of 0.9% NaCl was injected subcutaneously into the plantar side of the left hindpaw. The model was also used in rats, but 1 mg of zymosan A was used. Heat hyperalgesia was assessed 24 h and 6 h after induction of inflammation in mice and rats, respectively.

Neuropathic pain. Diazepam, L-838,417 and morphine were analysed in the CCI model[34] in 7–12-week-old mice or rats. Unilateral constriction injury of the left sciatic nerve just proximal to the trifurcation was performed with three loose ligatures. In sham-operated animals the sciatic nerve was exposed and the connective tissue was freed, but no ligatures were applied. In these sham-operated animals only a minor and transient hyperalgesia occurred. Heat hyperalgesia, cold allodynia and mechanical sensitization were assessed 7–9 days after surgery.

Heat hyperalgesia. Paw withdrawal latencies on exposure to a defined radiant heat stimulus were measured with a commercially available apparatus (Plantar Test; Ugo Basile). Four or five measurements were taken in each animal for every time point. Measurements of paw withdrawal latencies of the inflamed or injured paw and of the contralateral paw were made alternately.

Cold allodynia. The time spent lifting, shaking or licking the paw (seconds per minute) was measured for 5 min after application of a drop of acetone onto theaffected paw.

Mechanical sensitization. Mechanical sensitivity was assessed with electronic von Frey filaments (IITC). Triple measurements of paw withdrawal thresholds (g) were made for each time point and animal.

Locomotor activity. Locomotor activity was tested with a commercially available microprocessor-controlled activity cage (Actiframe; Gerb Elektronik). Mice were placed in the apparatus 15 min before testing. Motor activity was measured 10–30 min and 40–80 min after intrathecal and oral drug application, respectively.

Motor impairment. A possible impairment of motor function was assessed with the rotarod test[35]. Rats were trained on day zero and the maximum speed tolerated for at least 2 min

was determined for each rat. On the following day, rotarod performance was determined again 30 min after treatment with L-838,417 or vehicle (administered orally).

Electrophysiology. DRGs (from segments L4–L6) were removed from 14–24-day-old mice, dissociated and plated on poly-(L-lysine)-coated cover slips (for details see ref. 36). GABA-induced currents were recorded from capsaicin sensitive DRG neurons 3–30 h after plating. Transverse slices (250 μm thick) of the lumbar spinal cord were prepared from 14–24-day-old mice. GABAergic membrane currents were recorded from superficial dorsal horn neurons (laminae I and II) as described previously[3]. In both preparations, GABA (1 mM) was applied by short (10 ms) puffer applications to the soma of the recorded neuron at a frequency of 0.07 Hz. Diazepam (1 μM) was applied by means of bath perfusion. All recordings were made in the presence of the $GABA_B$ receptor antagonist CGP-55,845 (200 μM).

Immunofluorescence. The localization of $GABA_A$ receptor α subunits on primary afferent nerve terminals and intrinsic dorsal horn neurons was determined by double immunofluorescence staining on sections from perfusion-fixed adult mice[27]. Antibodies were home-made subunit-specific antisera[27] and commercial antibodies against substance P (T1609; Bachem) and NK1 (S8305; Sigma). Sections processed for double immunofluorescence were digitalized by confocal laser scanning microscopy (resolution 90 nm per pixel; two or three images per animal; n = 3 mice) and images were processed with Imaris (Bitplane). Double labeled objects (image profiles) in single confocal sections were identified by a segmentation algorithm (minimal size 0.2 μm^2; minimum intensity 50–90 on a 256-grey-level scale). The numbers of single-labelled and double-labelled profiles were calculated. All values are expressed as percentages of double-labelled profiles relative to the marker indicated.

Functional magnetic resonance imaging. fMRI experiments were performed in male Wistar rats weighing 350–400 g. During the measurements, rats were slightly anaesthetized with isoflurane (1–2 %) to maintain a respiration rate of about 60 c.p.m. and constant blood *p*CO2 levels. Measurements were made with a Bruker 4.7-T Biospec scanner with a free bore of 40 cm, equipped with an actively radio frequency decoupled coil system. A whole-body birdcage resonator enabled homogenous excitation, and a 3-cm quadrature surface coil, which served as a receiver, was located directly above the head of the animal to maximize the signal-to-noise ratio. Constant positioning of the rat's head within the scanner was verified by rapid acquisition of magnetic resonance images at 200-ms intervals. A functional series of 1,470 sets (4 s each, total of 96 min) of 22 axial images (slice thickness 1 mm, field of view 25 X 25 mm^2, 5.20 to -14.60 mm from the bregma[37]) were acquired with the

echo planar imaging technique (EPI: matrix 64 X 64, TR= 4,000 ms, TEef = 23.4 ms, two acquisitions). Anatomical scans with a high spatial resolution were obtained with RARE[38] (slice thickness 1 mm, field of view 25 X 25 mm^2, matrix 256 X 256, TR = 400 ms, TE = 18 ms, NEX = 8). Noxious heat stimulation was performed by applying temperature ramps (34–52 °C (noxious stimulation) or 34–42 °C (innocuous stimulation) with 15-s rise and fall times and a 5-s plateau phase) through two Peltier elements tightly attached to both hind paws (in awake rats this stimulation method yielded paw withdrawal latencies similar to those obtained in the behavioural tests with radiant heat). Thermal stimuli were applied to the left and right hind paw alternately at 2-min intervals. After 32 min of recording, L-838,417 (1 $mg \cdot kg^{-1}$) or vehicle was injected through an i.p. catheter without changing the position of the animal in the scanner. After drug injection, recording was continued for 64 min with the same stimulation method. Data were analysed with Brain voyager QX after appropriate preprocessing (motion correction, mean intensity adjustment, spatial smoothing 0.6 mm full-width at half-maximum, temporal gaussian smoothing 12 s, and temporal high-pass filtering of nine cycles) with a General Linear Modelling approach with four predictors: inflamed (left)/non-inflamed (right) paw before and after drug injection and Bonferroni correction. z-score maps of the individual rats weregroup analysed with custom-made analysis software (MagnAn[39] running under IDL). Anatomical and functional images were transferred into the register by an affine transformation scheme with only six degrees of freedom derived from the individual brain masks. The registered anatomical data and z-score maps were averaged over all animals. Contrast-specific mean z-score maps were calculated using a threshold of 3.0. Significantly activated voxels were labelled automatically with a digital standard rat brain atlas[37]. For each rat, brain structure and stimulation condition, we then first calculated the activation intensity as the stimulus-induced relative change in the BOLD signal (F). To quantify the effect of L-838,417 on the stimulus-induced BOLD signal changes we calculated $\Delta F/F$ as $(F_{post} - F_{pre})/F_{pre}$, where F_{post} is the value of F after drug treatment and F_{pre} is the value before drug treatment. Statistical analysis was performed witht he paired Student t-test. False-colour images of stimulus-induced changes in BOLD signals were obtained by mapping the calculated mean BOLD signal change of each voxel onto all significantly activated voxels. Note that the different colours in Fig. 7 encode F (signal amplitude), not statistical coefficients.

Results and Discussion

We first tested whether benzodiazepines exert antinociceptive effects at the level of the spinal cord by employing the mouse formalin assay, a model of tonic chemically induced pain. When the classical benzodiazepine diazepam was injected intrathecally into the lumbar spinal canal at doses of 0.01–0.09 mg per kg body weight, an apparent dose-dependent and

reversible antinociception was obtained that could be antagonized by systemic treatment with the benzodiazepine antagonist flumazenil (10 mg·kg^{-1} intraperitoneally (i.p.)) (Fig. 1).

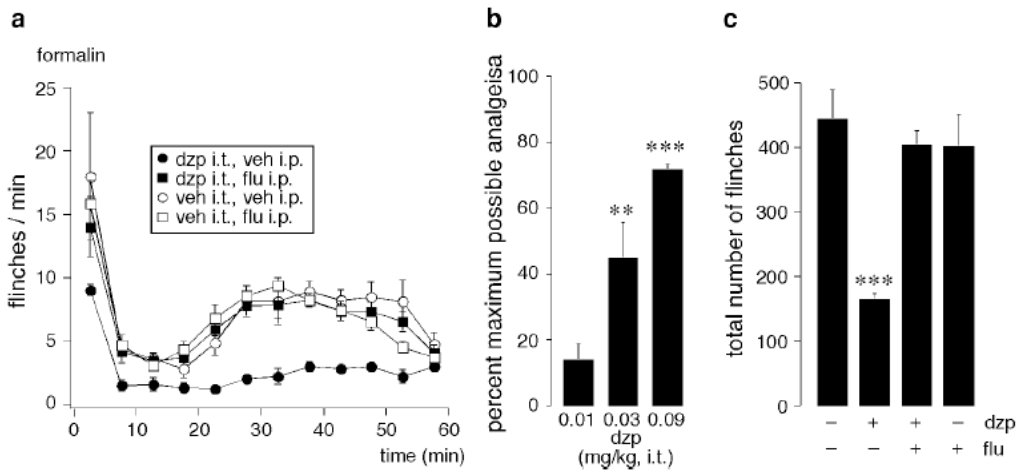

Figure 1. **Antinociceptive effects of diazepam (dzp) in the mouse formalin test.**

a, number of flinches (mean ± SEM, n = 5) versus time in wild-type (wt) mice treated with dzp 0.09 mg/kg i.t. and/or flumazenil (flu, 10 mg/kg, i.p.). **b,** maximum possible analgesia (number flinches in dzp-treated mice / number flinches in vehicle-treated mice x 100%) (mean ± SEM) obtained with different doses of i.t. dzp. **, statistically significant against vehicle with $P \leq 0.01$; ***, $P \leq 0.001$ (analysis of variance [ANOVA] followed by Bonferroni post hoc test). **c,** total number of flinches (mean ± SEM). ***, statistically significant against all other groups ($P \leq 0.001$, ANOVA followed by Bonferroni post-hoc test).

We next sought to identify the $GABA_A$ receptor isoforms responsible for this antinociception. $GABA_A$ receptors are heteropentameric ion channels composed from a repertoire of up to 19 subunits[16]. Benzodiazepine-sensitive isoforms are characterized by the presence of the $\gamma 2$ subunit and one of four α subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$)[17]. The generation of four lines of $GABA_A$-receptor point-mutated knock-in mice ($\alpha 1$(H101R), $\alpha 2$(H101R), $\alpha 3$(H126R) and $\alpha 5$(H105R)), in which a conserved histidine residue had been mutated to arginine, rendering the respective subunit insensitive to diazepam, has enabled the attribution of the different actions of diazepam to the individual $GABA_A$ receptor isoforms[10–12]. It also became possible to attribute the sedative effects of diazepam to $GABA_A$ receptors containing an $\alpha 1$ subunit[10] and the anxiolytic effect to those containing an $\alpha 2$ subunit[11] or - at high receptor occupancy - an $\alpha 3$ subunit[18]. We then compared the antinociceptive efficacy of intrathecal diazepam (0.09 mg·kg^{-1}) in wild-type mice with that obtained in the four types of $GABA_A$-receptor point-mutated mice in models of inflammatory hyperalgesia induced by subcutaneous injection of zymosan A in to one hindpaw and of neuropathic pain evoked by chronic constriction of the left sciatic nerve (chronic constriction injury (CCI) model). Wild-type mice and all four types of mutant mice developed nearly identical pain sensitization after induction of inflammation or

peripheral nerve injury (Fig. 2a, c). In wild-type mice, intrathecal diazepam (0.09 $mg \cdot kg^{-1}$) reversibly reduced inflammatory heat hyperalgesia (Fig. 2b), as well as CCI-induced heat hyperalgesia (Fig. 2d), cold allodynia (Fig. 2e) and mechanical sensitization (Fig. 2f) by 82 ± 13%, 92 ± 6% and 79 ± 9% (means ± s.e.m.), respectively. Responses of the non-inflamed or uninjured side were not significantly changed (Fig. 2a, c), indicating that spinal diazepam acted as an anti-hyperalgesic agent rather than as a general analgesic. Almost identical anti-hyperalgesic effects to those in wild-type mice were seen in mice carrying diazepam-insensitive α1 subunits. By contrast, α2(H101R) mice showed a pronounced reduction in diazepam-induced anti-hyperalgesia, which was consistently observed in all pain models tested. α3(H126R) and α5(H105R) mice showed smaller reductions, which occurred only in a subset of models. Importantly, intrathecal diazepam did not change spontaneous motor activity (Fig. 2g), indicating that the action of diazepam remained restricted to the spinal level and did not reach supra spinal sites, where sedation would have been induced. Anti-hyperalgesic effects of spinal diazepam can in principle originate from the facilitation of $GABA_A$ receptors at different sites. Diazepam might act either on postsynaptic $GABA_A$ receptors located on intrinsic dorsal horn neurons, thereby increasing postsynaptic inhibition, or on $GABA_A$ receptors located on the central terminals of primary afferent nerve fibres to increase primary afferent depolarization and presynaptic inhibition[19].

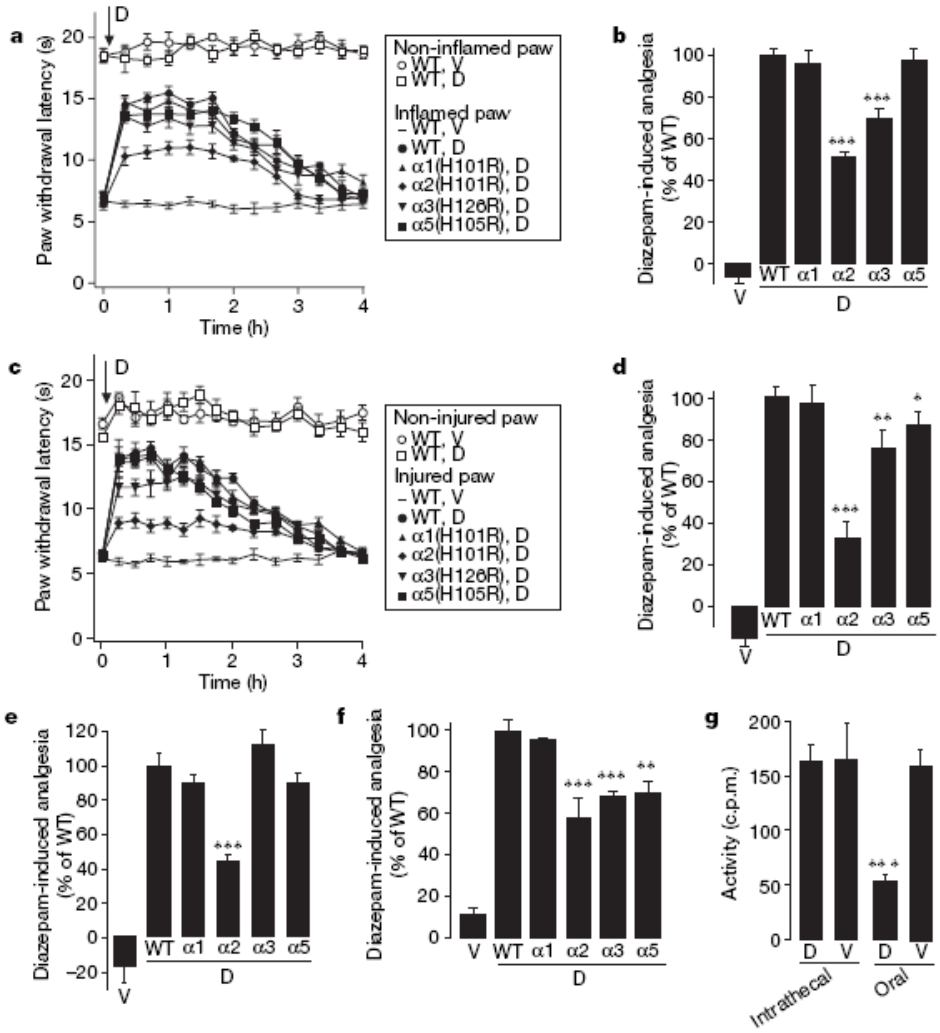

Figure 2. **Antinociceptive effects of spinal diazepam in different mouse pain models.**

a, b, Inflammatory pain induced by subcutaneous injection of zymosan A into the left hindpaw in wild-type (WT) mice and $GABA_A$ receptor point-mutated mice (α1(H101R), α2(H101R), α3(H126R), α5(H105R)). **a,** Paw withdrawal latencies (mean ± s.e.m.) in response to a defined radiant heat stimulus versus time after administration of intrathecal diazepam (D; 0.09 mg·kg^{-1}; arrowed) 48 h after injection of zymosan A. V, vehicle. **b,** Percentage diazepam-induced analgesia in the different genotypes. **c, d,** As in a and b, but for the CCI model of neuropathic pain. **e, f,** Effects of intrathecal diazepam (0.09 mg·kg^{-1}) on cold allodynia (e) and mechanical sensitivity (f) seven days after CCI surgery. Asterisk, P ≤ 0.05; two asterisks, P ≤ 0.01; three asterisks, P ≤ 0.001 (statistically significant against wild type; ANOVA followed by Bonferroni post-hoc test, n= 6 or 7 mice per group). **g,** Effects of diazepam (0.09 mg·kg^{-1} intrathecally, or 10 mg·kg^{-1} orally) on motor activity in the Actiframe test (mean ± s.e.m., n = 5 or 6), 10–30 min after intrathecal drug application or 40–80 min after oral drug application. Three asterisks, P ≤ 0.001 against vehicle (unpaired *t*-test).

To identify the benzodiazepine-sensitive $GABA_A$ receptor isoforms expressed at these sites we first employed electrophysiological measurements. GABAergic membrane currents were recorded from superficial dorsal horn neurons in transverse slices of spinal cords and from acutely isolated primary afferent (dorsal root ganglion (DRG)) nociceptive neurons characterized by their sensitivity to capsaicin. In nociceptive DRG neurons obtained from

α2(H101R) mice, the facilitation of GABAergic membrane currents by diazepam was completely abolished, whereas no significant alteration was found in neurons from α1(H101R), α3(H126R) and α5(H105R) mice (Fig. 3a). Facilitation of GABAergic membrane currents by diazepam in intrinsic superficial dorsal horn (lamina I/II) neurons was significantly decreased in α2(H101R) and α3(H126R) mice but not in α1(H101R) or α5(H105R) mice (Fig. 3b). We next employed confocal immunofluorescence microscopy of dorsal horn $GABA_A$ receptor α subunits and studied their colocalization with substance P (a marker for primary peptidergic nociceptors) and for neurokinin 1 (NK1) receptors (a marker for intrinsic nociceptive dorsal horn neurons in lamina I). Consistent with our electrophysiological experiments and with previous morphological results in the rat[20] was our observation that α2 and α3 were the most abundant diazepam-sensitive $GABA_A$ receptor α subunits in the mouse spinal dorsal horn (Fig. 4).

Co-staining experiments with antibodies against substance P or NK1 receptors (Fig. 3c–j and Table 1) revealed that α2, but not α1, α3 or α5, were extensively colocalized with substance-P positive primary afferent terminals in lamina II, whereas colocalization with NK1-receptor-positive lamina I neurons was greatest for the α3 subunit. Staining for α1 and α5 subunits was much less abundant and only occasionally colocalized with either substance P or NK1 receptors. Both sets of experiments indicate that intrinsic dorsal horn neurons express mainly $GABA_A$ receptor isoforms containing α2 and α3 subunits, whereas α2 is the dominant diazepam-sensitive $GABA_A$ receptor α subunit in adult DRG neurons (see also ref. 21).

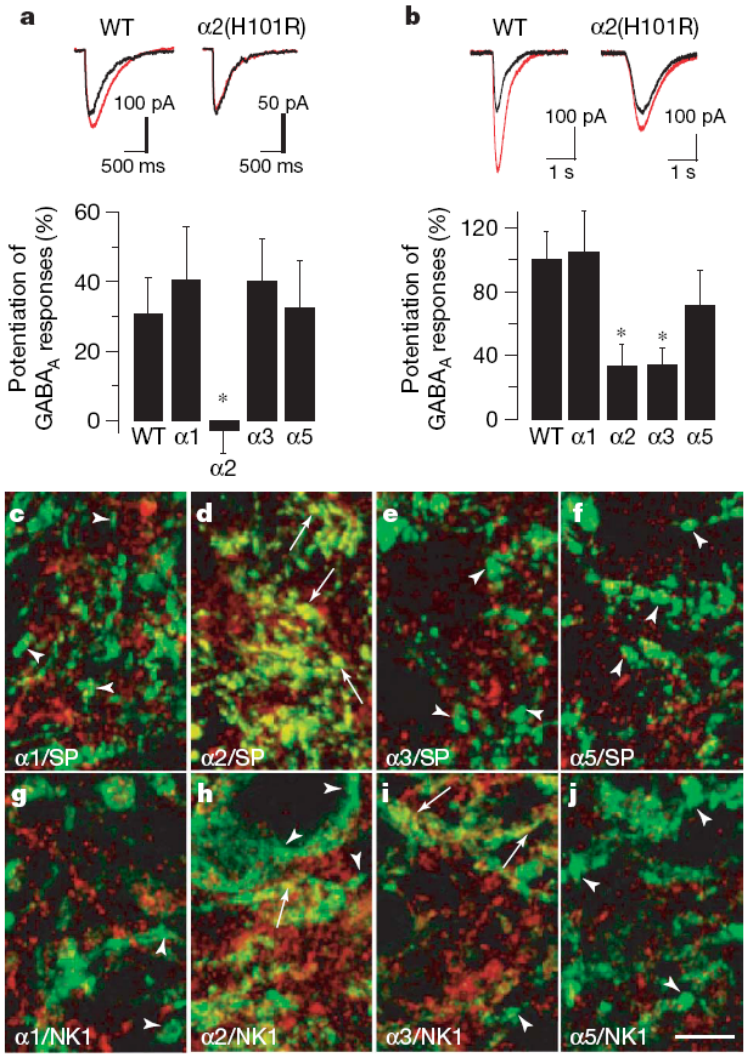

Figure 3. **GABA$_A$ receptor α subunits in capsaicin-sensitive primary afferent DRG neurons and in intrinsic dorsal horn neurons.**

a, b, Potentiation of GABAergic membrane currents by diazepam in wildtype (WT) and GABA$_A$ receptor mutant mice. **a,** DRG neurons. Averaged membrane currents evoked by puffer-applied exogenous GABA (1 mM) and percentage potentiation (mean ± s.e.m.) by diazepam (1 μM, n= 5–9). Asterisk, $P \leq 0.05$ (significant against all other genotypes; ANOVA followed by Fisher's post-hoc test). **b,** Intrinsic superficial dorsal horn neurons (mean s.e.m., n = 5–10). Asterisk, $P \leq 0.05$ (significant against wild-type and α1(H101R); ANOVA followed by Fisher's post-hoc test). **c–j,** Double immunofluorescence staining showing differential distribution of GABA$_A$ receptor α subunits (red) relative to substance P (SP)-positive axons and terminals (green) (c–f) or NK1 receptor-positive neurons **(g–j)** in laminae I and II. **c, g,** α1; **d, h,** α2; **e, i,** α3; **f, j,** α5. Arrows, double-labelled structures. Arrowheads, single-labelled structures devoid of GABA$_A$ receptor labelling. Scale bar, 5 μm **(c–j)**.

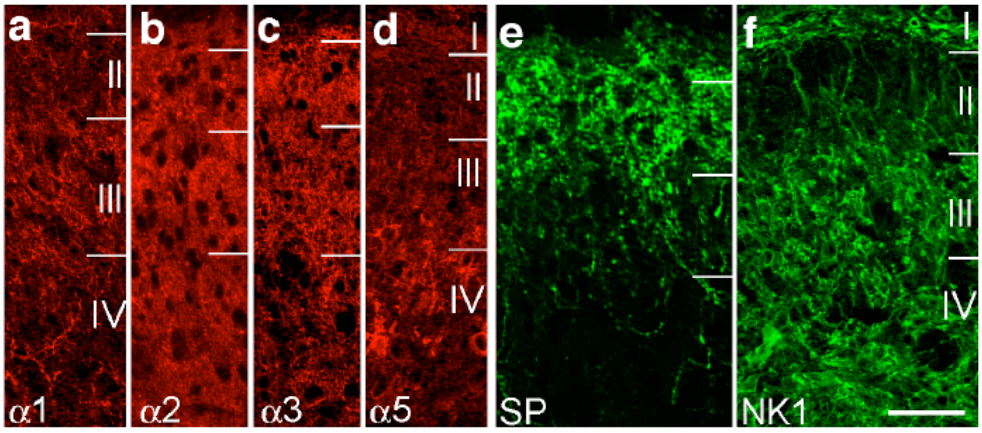

Figure 4. **Laminar distribution of $GABA_A$ receptor α subunits and of substance P (SP) and NK1 receptor immunoreactivity.**

Single immunofluorescence staining for the $GABA_A$ receptor α1, α 2, α 3, α5 subunits, SP, and NK1 receptors in lamina I and II of mouse lumbar spinal dorsal horn. **a – d,** distinct laminar distribution of $GABA_A$ receptor α subunits, illustrating the high relative abundance of α2 and α3 subunits. **e, f,** distribution of SP-positive primary afferent nerve fibers and NK1 receptor-positive neurons in the dorsal horn. Scale bar: 100 µm.

Table 1: Quantitative assessment of $GABA_A$ receptor subunit colocalization with substance P (SP) and NK1 receptors

markers	α2-SP/α2 α2α-SP/SP	α3-SP/α3 α3-SP/SP	α2-NK1/α2 α2-NK1/NK1	α3-NK1/α3 α3-NK1/NK1
co-localization	37±8% 47±13%	19±5% 24±5%	9±3% 27±6%	21±4% 47±9%

The decrease in diazepam-induced anti-hyperalgesia in α2(H101R) and α3(H126R) mice corresponds well to the presence of these subunits on primary afferent nerve terminals and/or on intrinsic dorsal horn neurons. So far, our results indicated that the spinal antinociceptive effect of diazepam is mainly mediated by $GABA_A$ receptor isoforms containing the α2 and α3 subunits, whereas the activation of α1-containing $GABA_A$ receptors is not involved. We therefore tested whether a similar analgesic effect would also be achieved after systemic treatment with subtype-selective benzodiazepine-site agonists, which spare the α1 subunit, by employing the non-sedative benzodiazepine-site ligand L-838,417, which is an antagonist at the α1 subunit and a partial agonist at receptors containing α2, α3 and α5 subunits[13]. Because L-838,417 possesses poor bioavailability and

an extremely short half-life in mice[22], it was tested in rats. After systemic treatment, L-838,417 produced dose-dependent and reversible anti-hyperalgesia in both the inflammatory and neuropathic pain models (Fig. 5). As expected, its maximum anti-hyperalgesic effect (Fig. 5a) was less than that of intrathecal diazepam, probably because L-838,417 exerts only partial agonistic activity. Anti-hyperalgesia was again completely reversed by flumazenil ($10mg \cdot kg^{-1}$ i.p.; Fig. 5b), indicating that it was mediated through the benzodiazepine-binding site of $GABA_A$ receptors. It was, however, insensitive to the opioid receptor antagonist naloxone ($10mg \cdot kg^{-1}$ i.p.), demonstrating that opioidergic pathways were not involved (Fig. 5b). L-838,417 did not impair motor coordination (Fig. 5c). We next investigated the effects of L-838,417 against neuropathic pain and compared its analgesic efficacy and its liability to tolerance development (that is, its loss of analgesic activity) with that of morphine. L-838,417 had a maximum analgesic effect comparable to that of morphine ($20mg \cdot kg^{-1}$ i.p.) (Fig. 5d), but unlike morphine it did not lose its efficacy during a chronic (nine-day) treatment period (Fig. 5 d, e).

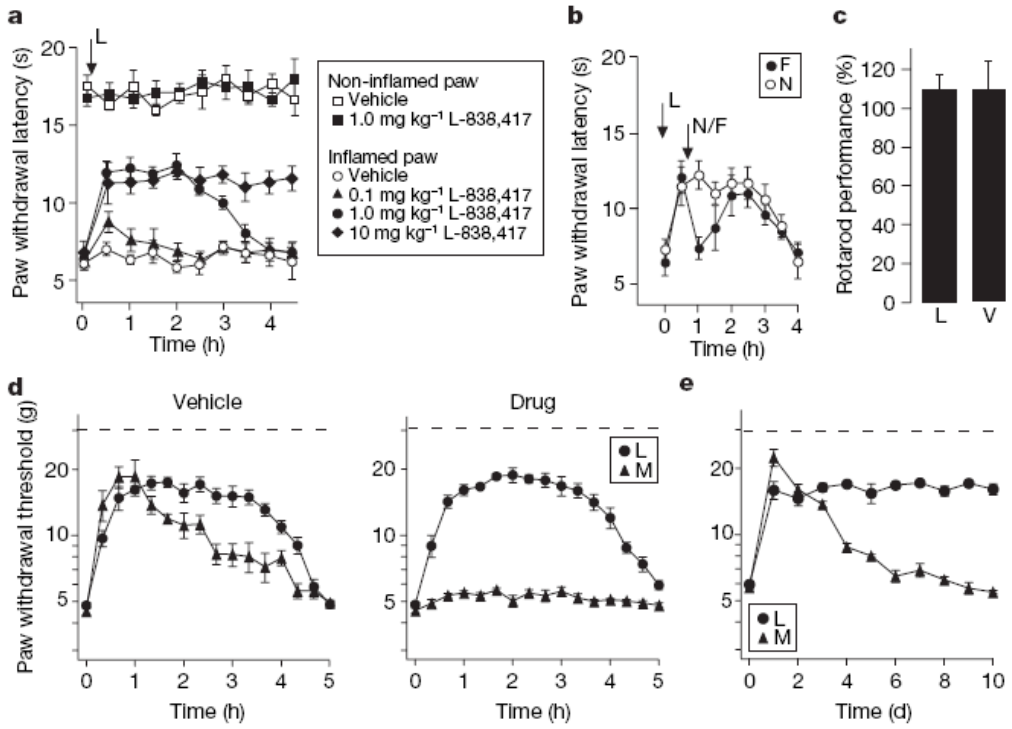

Figure 5. **Anti-hyperalgesic effects of the non-sedative benzodiazepine site ligand L-838,417 in rats.**

a, b, Inflammatory hyperalgesia induced by subcutaneous injection of zymosan A (1 mg) into one hindpaw. **a,** Effects of administration of L-838,417 (arrowed) on thermal hyperalgesia 6 h after injection of zymosan A (n = 4–6 rats). **b,** Effects of the benzodiazepine site antagonist flumazenil (F; 10 mg·kg^{-1} i.p.) and the opioid receptor antagonist naloxone (N; 10 mg·kg^{-1} i.p.) on antinociception induced by administration of L-838,417 (L; 1 mg·kg^{-1} orally). N = 3 rats per group. **c,** Effects of L-838,417 (1 mg·kg^{-1} orally) on motor control, shown as percentages of pre-drug rotarod performance (n = 8 rats per group). **d, e,** Neuropathic pain induced by CCI surgery. **d,** Anti-hyperalgesia by L-838,417 and morphine after chronic treatment (once-daily i.p. injections) for 9 days with either drug (right) or vehicle (left), 16 days after CCI surgery. Dashed lines, thresholds before CCI surgery. **e,** Analgesic efficacy of L-838,417 (L; 1 mg·kg^{-1}) and morphine (M; 20 mg·kg^{-1}) versus treatment duration. n = 6 rats per group. For a comparison with the anti-hyperalgesic activity of intrathecal diazepam in rats see Fig. 6. All data are means ± s.e.m.

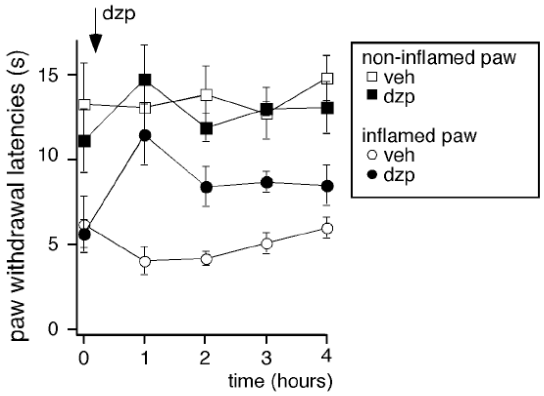

Figure 6. **Antihyperalgesic effects of intrathecal diazepam (dzp) in the rat.**

Inflammatory hyperalgesia was induced by subcutaneous injection of 1 mg zymosan A into the left hindpaw. Dzp (0.09 mg/kg) was injected i.t. and paw withdrawal latencies (mean ± sem, n = 5 / group) were measured 6 hrs post Zymosan A injection. The maximum analgesia achieved in rats was similar to that obtained mice, but shorter lasting.

Finally, functional magnetic resonance imaging (fMRI) was used to assess whether L-838,417 would reduce not only nociceptive behaviour but also the representation of pain in the central nervous system. Changes in blood-oxygenation-level-dependent (BOLD) signals were quantified to measure brain activation evoked by noxious heat. Stimulation of the inflamed left or the non-inflamed right hindpaw led to reliable, often bilateral, activation of several brain regions involved in pain processing (Fig. 7). Significantly more brain volume was activated and stronger activation was seen on stimulation of the inflamed paw. L-838,417 (1 $mg \cdot kg^{-1}$ i.p.) decreased brain activation induced by noxious heat after stimulation of the inflamed paw. For a quantitative assessment of its analgesic effects, we integrated the stimulus-correlated change in the BOLD signal (F) over all significantly activated voxels of each region of interest and calculated $\Delta F/F$ as $(F_{post} - F_{pre})/F_{pre}$, the relative decrease in F after injection of L-838,417 or vehicle (Table 2). Here we focused on brain areas that reflected either the sensory and discriminative component of pain (the medial thalamus and contralateral primary sensory cortex) or its emotional dimension (limbic system and frontal association cortex)[23,24]. After stimulation of the inflamed paw, a pronounced and statistically significant reduction in BOLD signal changes was observed in most brain regions analysed. Smaller changes in brain activation were found when the non-inflamed paw was stimulated and only negligible effects were seen after innocuous thermal stimulation (Table 2). These results indicate that systemically administered L-838,417 does indeed act as an anti-hyperalgesic agent and reduces BOLD signals in brain areas related to both the sensory and the emotional associative components of pain.

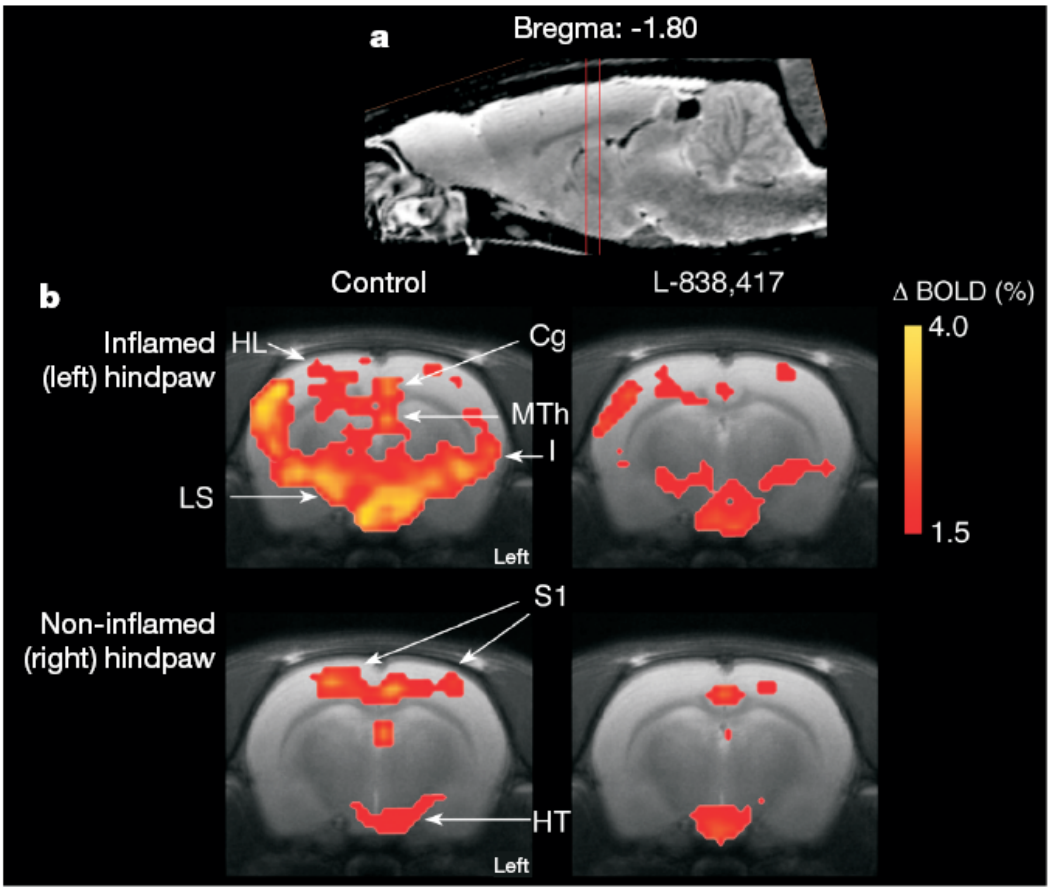

Figure 7. **Effects of L-838,417 (1 mg·kg^{-1} i.p.) on the supraspinal representation of pain.**

a, Anatomical slice indicating the position of the functional images. **b,** False-colour images of changes in BOLD signals evoked by stimulation of the left (inflamed) or right (non-inflamed) hindpaw with noxious heat. Images represent group maps across 12 rats averaged from 8 (pre-drug) and 16 (post-drug) stimulations. Experiments started 6 h after subcutaneous zymosan A injection into the left hindpaw. MTh, medial thalamus; S1, primary somatosensory cortex; Cg, cingulate cortex; I, insular cortex; LS, limbic system (including amygdala, entorhinal cortex and hippocampus); HT, hypothalamus; HL, representation of hindlimb in S1. Left, left hemisphere.

Table 2. **Changes in noxious heat-induced brain activation by L-838'417 measured by rat fMRI.**

	Stimulation of inflamed paw with noxious heat			**Stimulation of non-inflamed paw with noxious heat**			**Stimulation of non-inflamed paw with innocuous temperature**		
area	ΔF/F (mean±sem)	Incidence*	sign. vs. predrug (*P*)$^{+}$	ΔF/F (mean±sem)	Incidence*	sign. vs. predrug (*P*)$^{+}$	ΔF/F (mean±sem)	Incidence*	sign. vs. predrug (P)$^{+}$
MTh	-0.35 ± 0.07	10/12	0.014	-0.29 ± 0.09	10/12	0.069	-0.10 ± 0.07	6/12	0.302
S1c	-0.29 ± 0.07	12/12	0.028	-0.07 ± 0.09	12/12	0.383	-0.18 ± 0.25	11/12	0.228
Cg	-0.37 ± 0.07	11/12	0.034	-0.26 ± 0.08	12/12	0.078	-0.07 ± 0.08	10/12	0.152
FAC	-0.55 ± 0.05	12/12	0.007	-0.30 ± 0.08	12/12	0.179	-0.09 ± 0.08	6/12	0.253
LS	-0.36 ± 0.05	11/12	0.012	-0.06 ± 0.07	12/12	0.580	-0.04 ± 0.07	11/12	0.413

MTh, medial thalamus; S1c, primary somatosensory cortex contralateral; Cg, cingulate cortex; FAC, frontal association cortex; LS, limbic system (including amygdala, entorhinal cortex and hippocampus).

* number of rats, in which a significant noxious heat-induced activation of the respective area occurred / total number of rats studied

+ paired Student *t*-test

Considerable evidence indicates that a facilitation of GABAergic inhibition can be pronociceptive at supraspinal sites, for example the rostral agranular insular cortex[25] or in the periaqueductal grey[26], by reducing the activity of descending antinociceptive neurons. At these sites most $GABA_A$ receptors apparently contain the α1 subunit[27]. Therefore, not only would sparing the α1 subunit avoid unwanted sedation, it would also increase analgesic efficacy. Aside from sedation and tolerance development, addictive properties are of major concern in the development of analgesics. Available evidence indicates that subtype-selective benzodiazepine-site ligands should exhibit at most only modest addictive properties[28] and should not lead to tolerance development[29]. Finally, previous studies have shown that in neuropathic pain after injury to peripheral nerves, GABAergic inhibition can not only be diminished but it can even turn into excitation[6,7]. Our results suggest that sufficient inhibition remains to permit a spinal analgesic effect of drugs that increase GABAergic neurotransmission. Because glycine and GABA are released together at many inhibitory synapses in the dorsal horn[30], a facilitation of GABAergic transmission should also be able to compensate for a selective decrease in glycinergic inhibition[3]. Thus, we have not only identified the $GABA_A$ receptors containing the α2 and α3 subunits as critical components of spinal pain control, but also demonstrated that α1-sparing benzodiazepine-site ligands, which are already in development as anxioselective (non-sedative) agents, might constitute a class of analgesics suitable for the treatment of chronic pain syndromes.

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AUTHOR CONTRIBUTIONS

J.K., ***R.W.**, K.H., H.R. and U.B.Z. conducted the behavioural experiments. S.A. and J.B. made the electrophysiological recordings and analyses. M.S., A.H. and K.B. performed the fMRI study. J.M.F. made the morphological analyses. U.R. and H.M. provided the four lines of genetically modified mice. H.M. suggested experiments with L-838,417. H.U.Z. initiated the research, analysed behavioural and electrophysiological data and wrote the manuscript. All authors made comments on the manuscript.

***) Figures 2 e, f; 5 d, e; 6 (performance and analysis)**

3.2 Contribution of presynaptic $GABA_A$ receptors to the spinal control of nociception*

*__Witschi R__, Punnakkal P, Paul J, Fritschy JM, Keist R, Rudolph U, Zeilhofer HU, *submitted.*

Abstract

γ-amino butyric acid (GABA) is a major inhibitory neurotransmitter in spinal dorsal horn, a key site of nociceptive processing. Here, ionotropic GABA ($GABA_A$) receptors are expressed not only at classic postsynaptic sites, but also at the spinal terminals of primary afferent nociceptors where they contribute to presynaptic inhibition. Many of these presynaptic $GABA_A$ receptors belong to the α2 subunit containing type (α2-$GABA_A$ receptors), which is also largely responsible for the antihyperalgesic action of spinal benzodiazepines. The contribution of presynaptic α2-$GABA_A$ receptors to the spinal control of nociception and to the antihyperalgesic effect of spinal diazepam (dzp) is not known. Here, we address these questions through the use of conditional nociceptor-specific α2-$GABA_A$ receptor deficient (*sns*-α2$^{-/-}$) mice, and with conditional (*sns*-α2$^{R/-}$) point-mutated mice, whose primary nociceptor α2-$GABA_A$ receptor subunits have been rendered dzp-insensitive. We found that the amplitudes of $GABA_A$ receptor currents recorded from nociceptive dorsal root ganglion (DRG) neurons were unchanged in *sns*-α2$^{-/-}$ mice but their potentiation by dzp was significantly reduced. Accordingly, presynaptic inhibition of primary afferent evoked synaptic transmission in the superficial dorsal horn induced by the $GABA_A$ receptor agonist muscimol was unchanged in *sns*-α2$^{-/-}$ mice, but additional application of dzp-induced failures in synaptic transmission in wild-type mice only. In line with these *in vitro* results, behavioral experiments revealed unchanged nociceptive thresholds and unchanged inflammatory and neuropathic hyperalgesia in *sns*-α2$^{-/-}$ mice, but a decreased effect of spinal dzp against inflammatory hyperalgesia in *sns*-α2$^{-/-}$ (and *sns*-α2$^{R/-}$) mice. Our experiments did not reveal a contribution of presynaptic α2-$GABA_A$ receptors to endogenous pain control in mice but demonstrate a significant contribution of these receptors to dzp-induced anti-hyperalgesia in inflammatory pain states.

Introduction

$GABA_A$ receptors mediate fast synaptic inhibition throughout most parts of the adult mammalian central nervous system. They are also densely expressed in the spinal dorsal horn (1) where they control the propagation of nociceptive signals from the periphery to higher CNS areas (2, 3). Many lines of evidence indicate that diminished inhibitory GABAergic and glycinergic control at this site is a major factor in chronic pain syndromes ((4-8), for a review see (9)). Conversely, hyperalgesia originating from inflammatory and neuropathic disease states can be reversed by local spinal and systemic administration of the $GABA_A$ receptor agonist muscimol (10) or of benzodiazepines such as dzp (11, 12) and midazolam (13), which facilitate the action of GABA at $GABA_A$ receptors. These $GABA_A$ receptors are heteropentameric ligand-gated ion channels, composed of α, β and γ subunits

in a 2:2:1 stoichiometry (14, 15). All benzodiazepine-sensitive $GABA_A$ receptors contain one γ2 subunit, which together with an α1, α2, α3, or α5 subunit forms the benzodiazepine binding-site (16). $GABA_A$ receptor point-mutated mice have been generated in which each of the $GABA_A$ receptor α subunits has individually been rendered dzp-insensitive (17-19) with no change in the responses to the endogenous activator GABA. With these mice, it has become possible to attribute to α2-$GABA_A$ receptors not only the anxiolytic action of dzp (19), but also most of its spinal antihyperalgesic effect (11, 12).

These α2-$GABA_A$ receptors are not only found postsynaptically on central dorsal horn neurons, where they cause classic postsynaptic inhibition through hyperpolarization and activation of a shunting conductances, but a wealth of evidence indicates that they are also expressed presynaptically, on the spinal terminals of primary afferent nociceptive fibers (1). Due to a very peculiar expression pattern of chloride transporters (20, 21) and a resulting unusually high intracellular chloride concentration, $GABA_A$ receptor activation depolarizes rather than hyperpolarizes these terminals (22, 23) and give rise to a phenomenon known as primary afferent depolarization (PAD) (24, 25). This depolarization is nevertheless believed to cause presynaptic inhibition, i.e. reduce synaptic glutamate release from the spinal primary afferent terminals, most likely through voltage-dependent inactivation of presynaptic Na^+ or Ca^{2+} channels or through activation of a shunting conductance along the axon (26). However, if PAD becomes sufficiently strong to trigger action potentials, it may also cause so called dorsal root reflexes and exaggerate pain and inflammation (27, 28).

Although dorsal root potentials arising from the activation of presynaptic $GABA_A$ receptors in the spinal cord have been studied for decades, their functional contribution to the processing of nociceptive signals and to the antihyperalgesic effect of spinally applied benzodiazepines is still unknown, mainly due to lack of suitable tools for the specific targeting of presynaptic $GABA_A$ receptors. Here, we have used a genetic approach to address these questions and investigated conditional nociceptor-specific α2-$GABA_A$ receptor-deficient and point-mutated mice in morphological, electrophysiological and behavioral experiments. Nociceptor-specific α2-$GABA_A$ receptor deletion left GABAergic membrane currents in nociceptive DRG neurons and $GABA_A$ receptor-mediated presynaptic inhibition largely unchanged, but reduced their sensitivity to dzp and led to a reduction in the antihyperalgesic effect of spinally injected dzp.

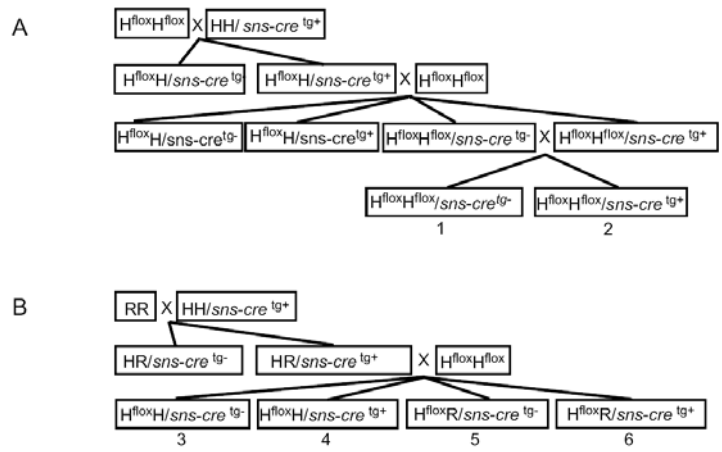
Materials and Methods

Mice. To generate a floxed *gabra2* allele, a 6.3 kb *PstI-NcoI* genomic fragment was isolated, which contains 2 *SphI* sites. The 1 kb *SphI-SphI* fragment was removed from the 6.3 kb *PstI-NcoI* fragment and replaced by an oligo hybrid containing a *loxP* site with adjacent *KpnI* and *SalI* sites, recreating a single *SphI* site, into which the 1 kb *SphI-SphI* fragment containing exon 5 (221 bp) was reinserted. A neomycine resistance cassette (*FRT-Pol2-neo-bpA-FRT-*

loxP) was then subcloned into the *SalI* site mentioned above. The genomic homology also contains exon 6 (83 bp). 3' adjacent to the genomic homology, a *HSV-TK* cassette was inserted. The vector backbone was pBC. The vector was linearized at the 5' end of the genomic homology at a *NotI* site. ES cells (C57BL/6N, Eurogentec) were electroporated and clones harboring a single targeting event (targeted allele) were obtained and injected into blastocysts (Polygene, Ruemlang, Switzerland), and germline transmission was obtained. The neomycine resistance cassette was bred out using *ACTFlpe* mice (Jackson Laboratories, Bar Harbor, Maine, stock number 005703) to obtain the floxed allele (*Gabra2*$^{tm2.1Uru}$.). Floxed mice were crossed with *EIIa*-cre mice (Jackson Laboratory, Bar Harbor, Maine, stock number 003724) to obtain *gabra2* global knockout mice. The designation of the knockout allele is: *gabra2*$^{tm2.2Uru}$.

Nociceptor-specific $GABA_A$ receptor α2-deficient or nociceptor-specific $GABA_A$ receptor α2 point-mutated mice were generated from *sns-cre* transgenic mice (29) crossed with $\alpha2^{fl/fl}$ or $\alpha2^{fl/fl}$ and $\alpha2^{R/R}$ (19) mice (for details of the breeding schemes see Fig. 1). All mice were maintained on a C57BL/6 background. To exclude possible confounding factors arising from the *sns-cre* transgene itself (41), we analyzed mice which carried the *sns-cre* transgene but no mutations in the *gabra2* gene. These mice developed normal inflammatory hyperalgesia and responded normally to intrathecal dzp (Fig. 6).

Mouse genotyping by polymerase chain reaction. The following polymerase chain reaction primers were used to identify the *cre* transgene (5'-TGA CAG CAA TGC TGT TTC ACT GG-3' and 5'-GCA TGA TCT CCG GTA TTG AAA CTC C-3', providing a product size of 607 bp), the $GABA_A$ α2 point-mutated α2(H101R; R) allele (5'- TCC ATC ATC CTG GAT TCG AAG CAG C-3' and 5'-GCA TGC ACC ACC CAG GAA GCG ATT-3', providing a product size of 526 bp for the wildtype (H) or floxed (H^{flox}) allele and 561 bp for the point-mutated (R) allele), and the floxed $GABA_A$ α2 allele (5'- TAT CTT GTC TTT CCC CTC CTG GTT G-3' and 5'- CAG GAT AGG GAA GCA GGA GTG G-3', providing a product size of 289 bp for the wildtype (H) or point-mutated (R) allele and around 330 bp for the floxed α2 allele. The regions amplified for the floxed allele and for the α2(H101R) allele are separated.



C

	Gabra2 alleles	*sns-cre* ^{tg}	combined genotype	nociceptors	rest	Short notation
1	$H^{flox}H^{flox}$	-	$H^{flox}H^{flox}$/*sns-cre* $^{tg-}$	H/H	H/H	$\alpha2^{fl/fl}$ pseudo wt
2	$H^{flox}H^{flox}$	+	$H^{flox}H^{flox}$/*sns-cre* $^{tg+}$	-/-	H/H	*sns*-$\alpha2^{-/-}$
3	$H^{flox}H$	-	$H^{flox}H$/*sns-cre* $^{tg-}$	H/H	H/H	pseudo wt
4	$H^{flox}H$	+	$H^{flox}H$/*sns-cre* $^{tg+}$	-/H	H/H	*sns*- $\alpha2^{-/H}$
5	$H^{flox}R$	-	$H^{flox}R$/*sns-cre* $^{tg-}$	H/R	H/R	$\alpha2^{H/R}$
6	$H^{flox}R$	+	$H^{flox}R$/*sns-cre* $^{tg+}$	-/R	H/R	*sns*-$\alpha2^{R/-}$

Figure 1. **Breeding schemes and description of the genotypes of mice used in the present study.**

A, breeding scheme to obtain nociceptor-specific $\alpha2^{-/-}$ ($H^{flox}H^{flox}$/*sns-cre*$^{tg+}$) mice and their pseudo-wild-type litter mates ($\alpha2^{fl/fl}$)**. B,** breeding scheme to obtain nociceptor-specific $\alpha2^{R/-}$ ($H^{flox}R$/*sns-cre*$^{tg+}$), $\alpha2^{-/H}$ ($H^{flox}H$/*sns-cre*$^{tg-}$), and corresponding control mice (global heterozygous and pseudo-wild type). **C,** genotypes of all mouse lines. H^{flox}, $\alpha2$ floxed allele; H, $\alpha2$ wild-type allele with a codon for histidine at amino acid position 101; R, $\alpha2$(H101R) point-mutated allele with a codon for arginine at amino acid position 101; *sns-cre*^{tg}, absence (-) or presence (+) of *cre* transgene.

Gene expression analysis by real-time PCR. Lumbar dorsal root ganglia (4 per tube), lumbar spinal cords and cerebral cortices from adult (7-10 weeks) *sns*-$\alpha2^{-/-}$ mice and $\alpha2^{fl/fl}$ littermates, and from global $\alpha2^{-/-}$ mice, were rapidly removed following decapitation of the mice in ice-cold carbogen-saturated extracellular solution (see more below). Biopsies were collected in a tube containing a lysis solution and total RNA was isolated by using GenElute™ Mammalian Total RNA Miniprep Kit (RTN70, Sigma-Aldrich). gDNA was removed and cDNA was generated in a reaction mixture using QuantiTect® Reverse Transcription Kit (Cat.No. 205311, Qiagen®). Real-time quantitative PCR (qPCR) was performed analyzed using TaqMan® Gene Expression Master Mix (Part No. 4369016, Applied Biosystems) with generated cDNA in a total reaction volume of 10 µl in 384-Well arrays with 7900HT Fast Real-Time PCR System and software (Applied Biosystems). mRNA expression of the benzodiazepine-sensitive $GABA_A$ receptor α subunits ($\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$) was quantified using pre-designed coding assays (FAM™ dye-labeled TaqMan® MGB probes; Applied Biosystems) (Table 1). The Real-time qPCR cycling program consisted of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The expression level of each targeted gene was normalized to that of the β-actin gene, which was used as a reference. All qPCR reactions were carried out in triplicate. Relative quantification of transcript was determined using the comparative CT method ($2^{-\Delta CT}$) calibrated to β-actin.

Table 1. **Assay probes used in the qRT-PCR analysis.**

Assay ID	Context Sequence	Gene	Gene Name	Species	Exon Boundary
Mm00607939_s1*	CTGTTACTG AGCTGCGT TTTACACC	*Actb*	actin, beta, cytoplasmic (reference gene)	Mus musc.	6
Mm00439046_m1**	TTCCAGAAA AGCCAAAG AAAGTAAA	*Gabra1*	$GABA_A$ receptor, subunit alpha 1	Mus musc.	9-10
Mm00433435_m1**	TATATACCA TGAGGCTT ACAGTCCA	*Gabra2*	$GABA_A$ receptor, subunit alpha 2	Mus musc.	5-6
Mm00433440_m1**	AGTGACTG TGACACTC GATCTCAC A	*Gabra3*	$GABA_A$ receptor, subunit alpha 3	Mus musc.	1-2
Mm00621092_m1**	ACACCATG CGTCTGAC AATCTCTGC	*Gabra5*	$GABA_A$ receptor, subunit alpha 5	Mus musc.	5-6

*) The assay's primers and probes are designed within a single exon and will detect genomic DNA.

**) The assay's probe spans an exon junction and will not detect genomic DNA.

Preparation of spinal cord slices for electrophyiological experiments. 2 - 3 week-old mice were anesthetized with isoflurane and subsequently sacrificed by decapitation. The spinal cord was removed after laminectomy in ice-cold extracellular solution, fixed on gelatine block and were sliced into transverse sections (300-350 μm) with few dorsal roots. Slices were kept in extracellular solution 120 mM NaCl, 5 mM Na-HEPES, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose (pH 7.35, 300 mOsm) at physiological temperature around 35°C and bubbled with 95% O_2 / 5% CO_2.

Electrophysiology. DRG neurons were prepared from 3 - 4 week old *sns*-$\alpha2^{-/-}$ and $\alpha2^{fl/fl}$ mice as described previously (11). GABAergic membrane currents and their potentiation by dzp were studied in nociceptive DRG neurons identified through the presence of Na^+ current remaining in the presence of TTX (0.3 μM) (31, 42, 43). Spinal cord slices were prepared from 2 - 3 week-old mice (7). Primary afferent EPSCs were elicited at a frequency of 0.07 Hz through electrical stimulation of short dorsal rootlets attached to the spinal cord slices, and recorded from LI/II neurons. Cl^- was replaced by F^- in the intracellular solution to avoid the activation of $GABA_A$ receptor-meditated membrane currents in the recorded neuron during perfusion with muscimol. All recordings were made at room temperature.

Behavior. Experiments were done in 7-10-week old mice of both sexes. Inflammatory and neuropathic pain induction, as well as thermal and mechanical testing was done as described previously (11). Acute C fiber-mediated nociception was tested following subcutaneous capsaicin (1.6 μg / 10 μl vehicle [10% Tween 80, 10 % ethanol, 80 % saline]) injection and flexor responses (flinches) were counted for 5 min following injection. C-fiber-dependent secondary hyperalgesia was induced through capsaicin (30 μg / 10 μl vehicle [10% Tween 80, 10 % ethanol, 80 % saline] injection into the plantar side of the left hind paw (44). Permission for the animal experiments was obtained from the Veterinäramt des Kantons Zürich (ref. no. 121/2006 and 34/2007).

Morphology. Lumbar spinal cords prepared from 6 - 8 week old *sns*-$\alpha2^{-/-}$ mice and $\alpha2^{fl/fl}$ littermates were cut into 300 μm thick parasagittal slices, fixed in 4% paraformaldehyde for 10 min and subsequently cut into 14 μm thick sections using a cryostat. Immunofluorescence stainings were made to study the co-localization of $GABA_A$ receptor $\alpha2$ and $\alpha3$ subunits (guinea pig affinity purified antisera; (11)) with markers of primary afferent fibers. Polyclonal rabbit antisera against CGRP (Chemicon, California, USA, cat. no. AB 15360) and an isolectin B4 (IB4)-Alexa 488 conjugate (Molecular Probes, Eugene, OR, USA, cat. no. 121411) were used to label the spinal terminals of peptidergic and non-peptidergic C fibers, respectively. Myelinated fibers were retrogradely labeled with cholera toxin B subunit (CTB,

Sigma-Aldrich, St. Louis, MO, USA, 2 µg) injected slowly (within 10 min) in a volume of 2 µl into the left sciatic nerve proximal to its trifurcation. 7 days after injection, mice were sacrificed. CTB was detected with goat anti-CTB serum (List Biological Laboratories, Campbell, CA, cat. no. 703). High-resolution confocal images were processed and analyzed with Imaris (Bitplane).

Results

Conditional nociceptor-specific α2-GABA$_A$ receptor-deficient mice (*gabra2* $H^{flox}H^{flox}$/*sns*-cre^{tg+}; short *sns*-$\alpha2^{-/-}$ mice) were generated by crossing mice carrying a floxed α2-GABA$_A$ receptor (*gabra2*) gene (Fig. 1 and 2A; for details see Materials and Methods) to bacterial artificial chromosome transgenic mice expressing the *cre* combinase under the transcriptional control of the sensory neuron specific sodium channel (*sns*) gene (29). To quantify changes in GABA$_A$ receptor α2 subunit expression and to test for possible compensatory up- or down-regulation of other benzodiazepine-sensitive GABA$_A$ receptor α subunits, we first employed qRT-PCR (detecting the floxed *gabra2* gene segment) in lumbar DRGs and spinal cords, and cerebral cortices (Fig. 2B,C). Compared to $\alpha2^{fl/fl}$ mice, *sns*-$\alpha2^{-/-}$ mice showed a 66.2 ± 1.3% (n = 7) reduction in GABA$_A$ receptor α2 subunit mRNA expression with no significant changes in spinal cord and cerebral cortex (Fig. 2B). The expression of the other benzodiazepine-sensitive GABA$_A$ receptor α subunits was not significantly changed in DRGs of *sns*-$\alpha2^{-/-}$ mice (Fig. 2C). No detectable levels of α2 subunit mRNA were found in global $\alpha2^{-/-}$ mice (which were generated from the same $\alpha2^{fl/fl}$ mice through *EIIa-cre* mice (30) suggesting that the α2 mRNA levels remaining in the *sns*-$\alpha2^{-/-}$ mice derived most likely from non-nociceptive (*sns-cre*-negative) DRG neurons.

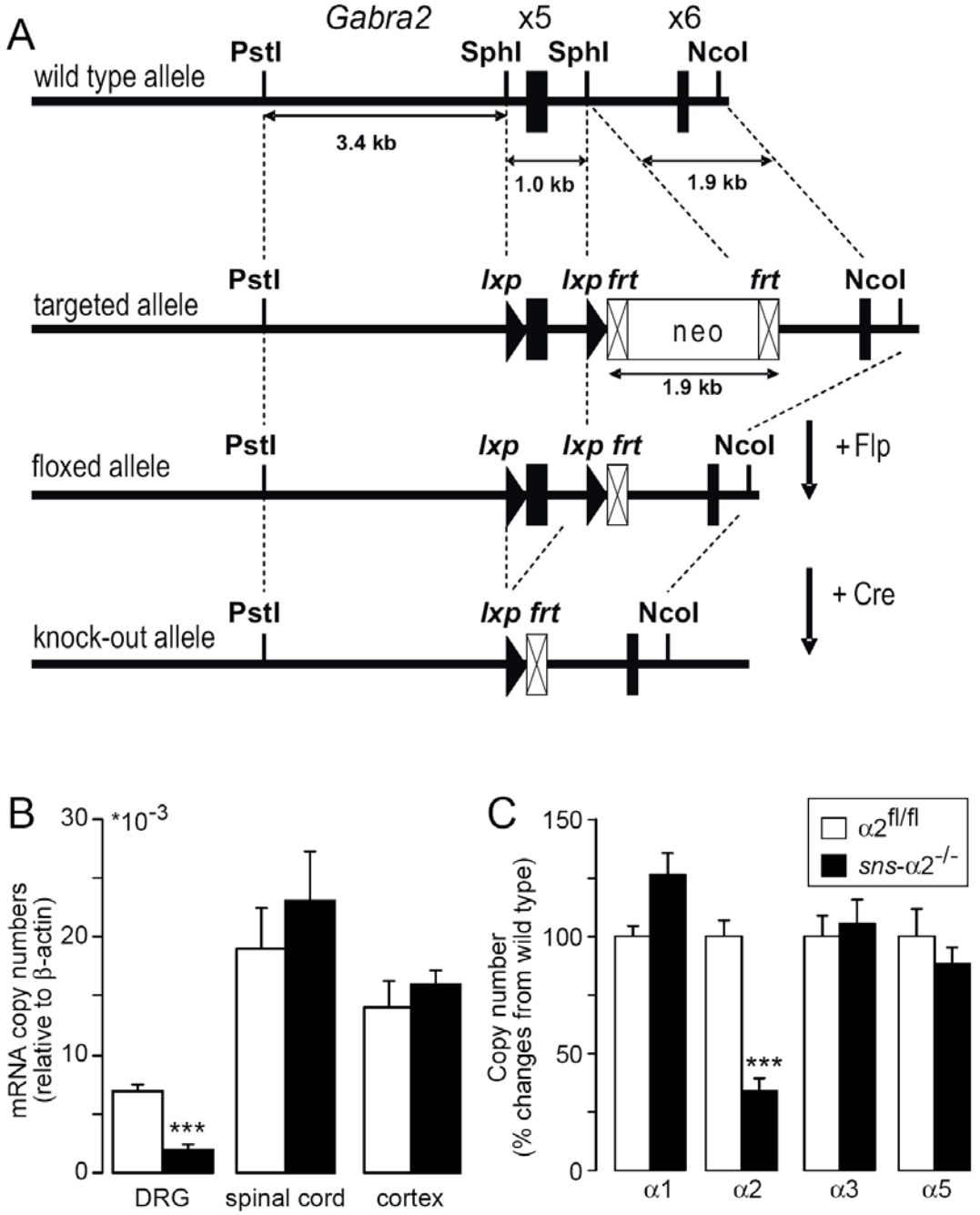


Figure 2. **Generation of $GABA_A$ receptor $\alpha 2^{fl/fl}$ mice and qRT-PCR analyses.**

(A) Generation of mice carrying a floxed *gabra2* allele . Removal of the *Neo* cassette, flanked by flippase recognition targets (*frt*) by the flippase (*Flp*) and the removal of the flanked region by *loxP* sites by the *Cre* recombinase, respectively. The two bars show exon 5 (x5) and 6 (x6). (B) Quantification (mean ± sem) of *gabra2* transcript numbers (relative to β-actin) in lumbar DRGs, spinal cords and cerebral cortices of *sns*-$\alpha 2^{-/-}$ mice (n = 7) and wild type ($\alpha 2^{fl/fl}$) littermates (n = 9) with qRT-PCR. (C) Quantitative analysis of changes in the copy number of *gabra1*, *gabra2*, *gabra3* and *gabra5* gene transcripts (encoding for the benzodiazepine-sensitive subunits α1, α2, α3, and α5) in the DRGs of *sns*-$\alpha 2^{-/-}$ mice and wild type ($\alpha 2^{fl/fl}$) littermates. ***, P ≤ 0.001 Statistical comparisons between wild-type and *sns*-$\alpha 2^{-/-}$ mice were made with Student *t* tests followed by Bonferroni correction for three (B) and four (C) independent comparisons.

High resolution confocal microscopy was used in coronal section of the lumber spinal cord to study the co-localization of α2-GABA$_A$ receptors with calcitonin gene related peptide (CGRP) and isolectin B4 (IB4), two markers of peptidergic and non-peptidergic C fiber nociceptors, respectively (Fig. 3A). In dorsal horn layers I and II of $\alpha2^{fl/fl}$ mice, about one third and one fourth of the CGRP and IB4 positive axon terminals also stained positive for α2-GABA$_A$ receptors. These co-localizations were dramatically reduced in *sns*-$\alpha2^{-/-}$ mice (Fig. 3B). To study co-localizations in non-nociceptive (myelinated) axons, we labeled these fibers retrogradely through injection of cholera toxin B subunit (CTB) into the sciatic nerve of anesthetized mice. CTB-positive structures were mainly located in the deep dorsal horn and showed only very little co-localization with α2-GABA$_A$ receptors. Interestingly, much of the total α2 immunoreactivity was retained in *sns*-$\alpha2^{-/-}$ mice, which together with the low expression of α2 in myelinated primary afferents, indicates that a major portion of dorsal horn α2-GABA$_A$ receptors reside on central neurons rather than on primary afferent terminals. Rather unexpectedly, we found similar degrees of co-localization with CGRP- and IB4-positve terminals also for α3-GABA$_A$ receptors (Table 2).

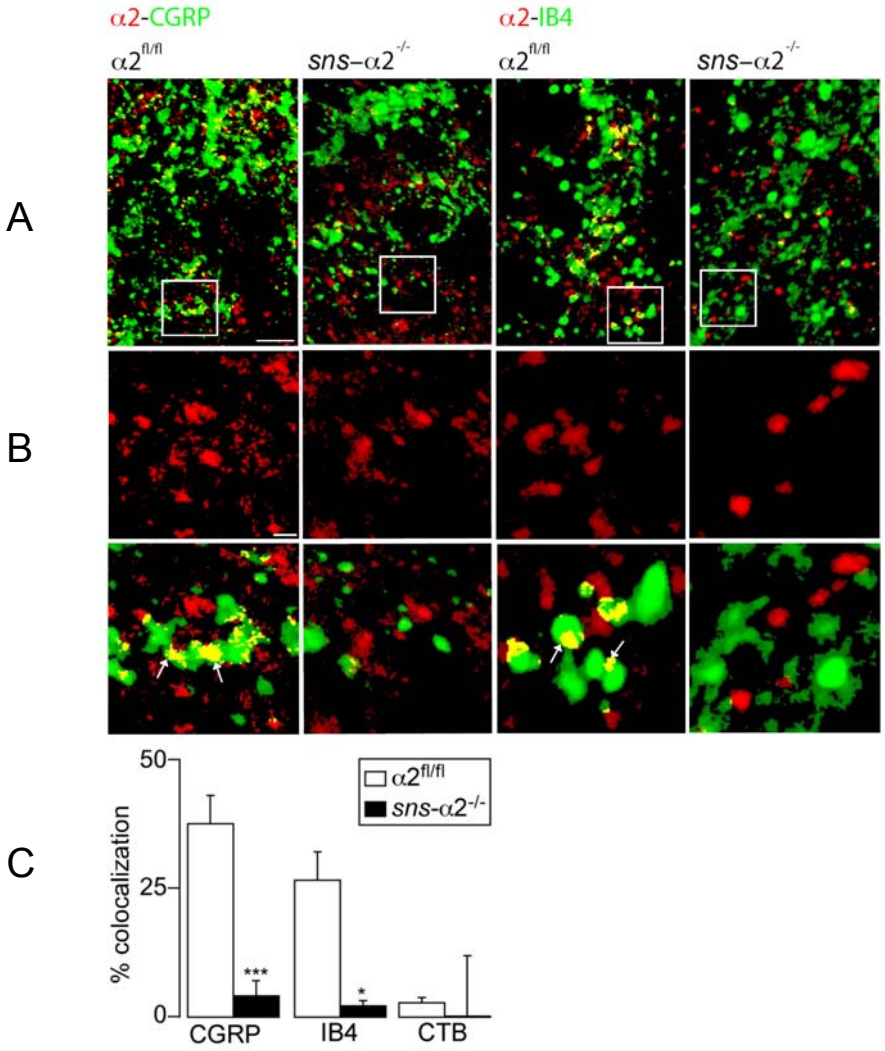


***Figure 3.* α2-GABA$_A$ receptors in the spinal dorsal horn.**

(A) Double immunofluorescence stainings showing co-localization of α2-GABA$_A$ receptors (red) with peptidergic (CGRP-positive, lamina II outer) and non-peptidergic (IB4-positive, lamina II inner) axons and terminals (green) in parasagittal sections of lumbar spinal cords of adult wild type ($\alpha2^{fl/fl}$) and *sns*-$\alpha2^{-/-}$ mice. Scale bar, 5 µm. (B,C) Higher magnification of the areas indicated in (A). Scale bars, 0.5 µm. (B) α2-GABA$_A$ receptor immunoreactivity. (C) Co-localization. Arrows indicate co-localizations. (D) Statistical analysis. Per cent co-localization (mean ± sd) of CGRP, IB4 and CTB-positive axons and terminals with α2-GABA$_A$ receptors. Co-localizations were counted in 6 fields per slide. Each slide was from different mouse and 3 mice per genotype were analyzed. ANOVA followed by Bonferroni post hoc test $F(5,12) = 47.0$; ***, $P \leq 0.001$. Co-localization of CTB with α2-GABA$_A$ receptors was assessed in lamina III of the dorsal horn.

Table 2. **Percentage colocalization of α3 clusters with the primary afferent terminals.**

	CGRP			IB4			CTB		
	%	SD	SEM	%	SD	SEM	%	SD	SEM
$\alpha 2^{fl/fl}$	52.2	11.88	6.86	40.6	15.82	9.14	3.0	5.37	3.10
***sns*-$\alpha 2^{-/-}$**	43.5	9.01	5.20	41.0	33.41	19.29	0.5	0.87	0.50

To analyze functional consequences of *sns*-α2 gene deletion for GABAergic membrane currents, we made whole-cell recordings from acutely isolated nociceptive DRG neurons identified by the presence of Na^+ currents remaining in the presence of tetrodotoxin (TTX, 0.3 μM) (31). No significant changes in the amplitude of GABAergic membrane currents were found between DRG neurons isolated from *sns*-$\alpha 2^{-/-}$ mice and $\alpha 2^{fl/fl}$ littermates, but the facilitation of GABAergic currents by dzp (1 μM) was significantly reduced in the *sns*-$\alpha 2^{-/-}$ group (Fig. 4A). We next analyzed the modulation of primary afferent-evoked synaptic transmission through presynaptic $GABA_A$ receptors. In *sns*-$\alpha 2^{-/-}$ mice, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated excitatory postsynaptic currents (EPSC) were recorded from superficial (lamina I/II) dorsal horn neurons upon electrical stimulation of short dorsal roots.

To avoid the activation of $GABA_A$ receptors in the recorded neurons, intracellular Cl^- was replaced by F^- (32), which does not permeate $GABA_A$ gated Cl^- channels (33). Amplitudes of AMPA-EPSCs were not significantly different between neurons recorded from *sns*-$\alpha 2^{-/-}$ and $\alpha 2^{fl/fl}$ littermates and similarly decreased by muscimol (0.1 μM and 5 μM) (figure 4B). We next studied the effect of dzp on the inhibitory action of a low concentration of muscimol (0.1 μM). Muscimol alone had only a minor effect on AMPA-EPSCs, but when dzp (1 μM) was applied in addition to muscimol, the rate of "successful" stimulation decreased significantly (Fig. 4C) consistent with a presynaptic site of action.

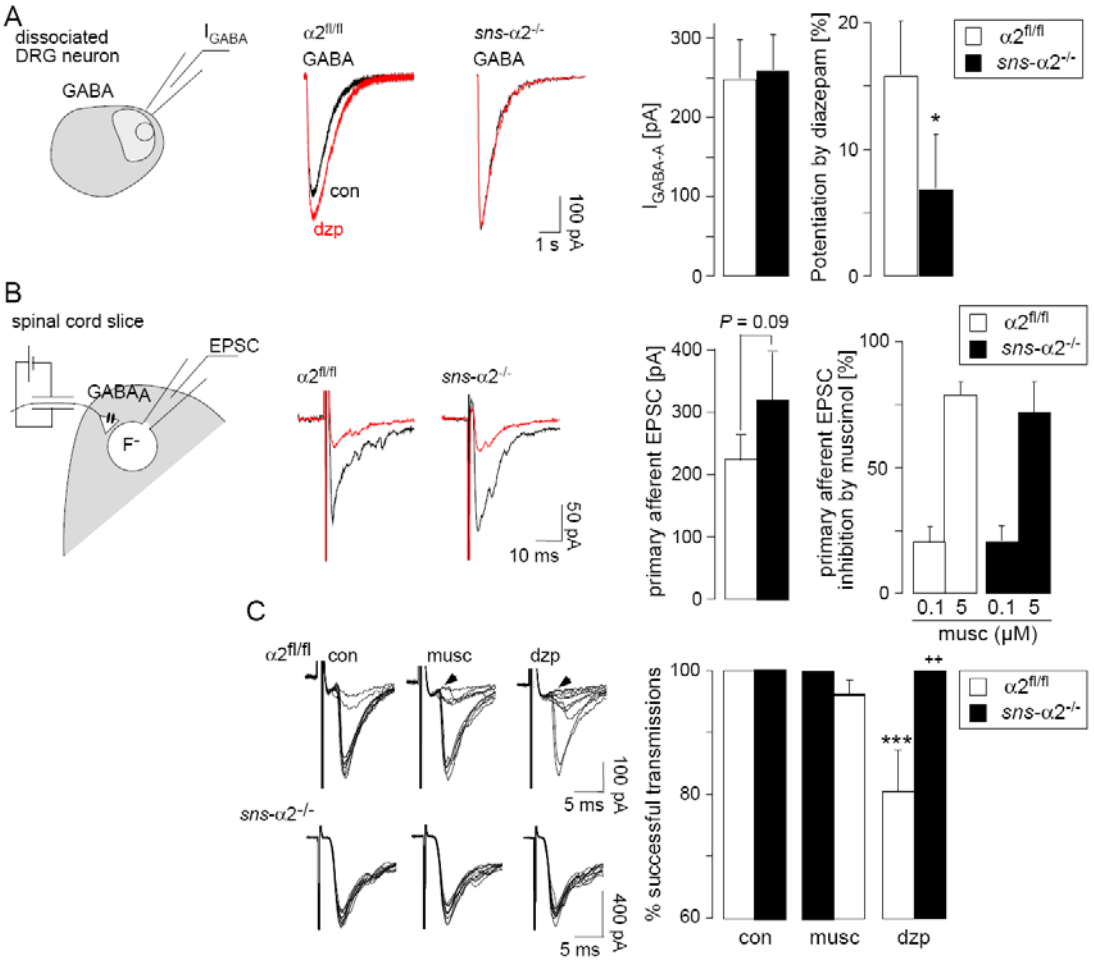


Figure 4. **GABAergic membrane currents and primary afferent synaptic transmission in *sns*-α2$^{-/-}$ mice.**

(A) GABAergic membrane currents recorded from acutely isolated nociceptive DRG neurons. Left: individual current traces evoked through puffer application of GABA (1 mM) to the soma of DRG neurons of $\alpha2^{fl/fl}$ (n = 26) and *sns*-$\alpha2^{-/-}$ mice (n = 14) and recorded in the absence (black) and presence (red) of dzp (1 μM). Right: statistical analyses (mean ± sem). Absolute amplitudes and percent potentiation by dzp. *, $P < 0.05$ (unpaired t test). (B, C) Primary afferent evoked EPSCs recorded from lamina I/II neurons in coronal spinal cord slices of $\alpha2^{fl/fl}$ and *sns*-$\alpha2^{-/-}$ mice. (B) left: current traces under control conditions (black) and in the presence of muscimol (5 μM, red). Right: statistical analyses (mean ± sem). EPSC amplitudes: unpaired t test, n = 19 ($\alpha2^{fl/fl}$), n = 18 (*sns*-$\alpha2^{-/-}$); inhibition by muscimol: n = 6 - 17. (C) Analyses of synaptic failure rates. Left: superposition of 10 consecutive primary afferent-evoked EPSCs under control conditions, in the presence of muscimol (0.1 μM) and in the additional presence of dzp (1 μM). Right: statistical analysis (mean ± sem). n = 17 ($\alpha2^{fl/fl}$), n = 10 (*sns*-$\alpha2^{-/-}$). ANOVA (genotype * treatment); F (3,81) = 3.96; P = 0.03; **, $P < 0.01$ significant against $\alpha2^{fl/fl}$; +++, $P < 0.01$ significant against control.

We next analyzed possible phenotypes of *sns*-$\alpha 2^{-/-}$ mice in tests of acute nociception and in models of inflammatory and neuropathic hyperalgesia. *sns*-$\alpha 2^{-/-}$ mice responded normally to exposure of one hindpaw to noxious heat or to mechanical stimulation with von Frey filaments. They also exhibited normal nociceptive (flexor) responses (flinches) after chemical activation of nociceptors with subcutaneously injected capsaicin (Fig. 5A). Next, we analyzed the mice in inflammatory and neuropathic pain models. *sns*-$\alpha 2^{-/-}$ and $\alpha 2^{fl/fl}$ mice developed virtually identical thermal and mechanical hyperalgesia (Fig. 5B,C) and indistinguishable inflammatory paw swelling (Fig. 5D) after subcutaneous injection of the yeast extract zymosan A. Likewise, *sns*-$\alpha 2^{-/-}$ and $\alpha 2^{fl/fl}$ mice responded with nearly identical thermal and mechanical hyperalgesia after chronic constriction of the sciatic nerve (Fig. 5E,F), and developed normal mechanical hyperalgesia after subcutaneous capsaicin injection (Fig. 5G).

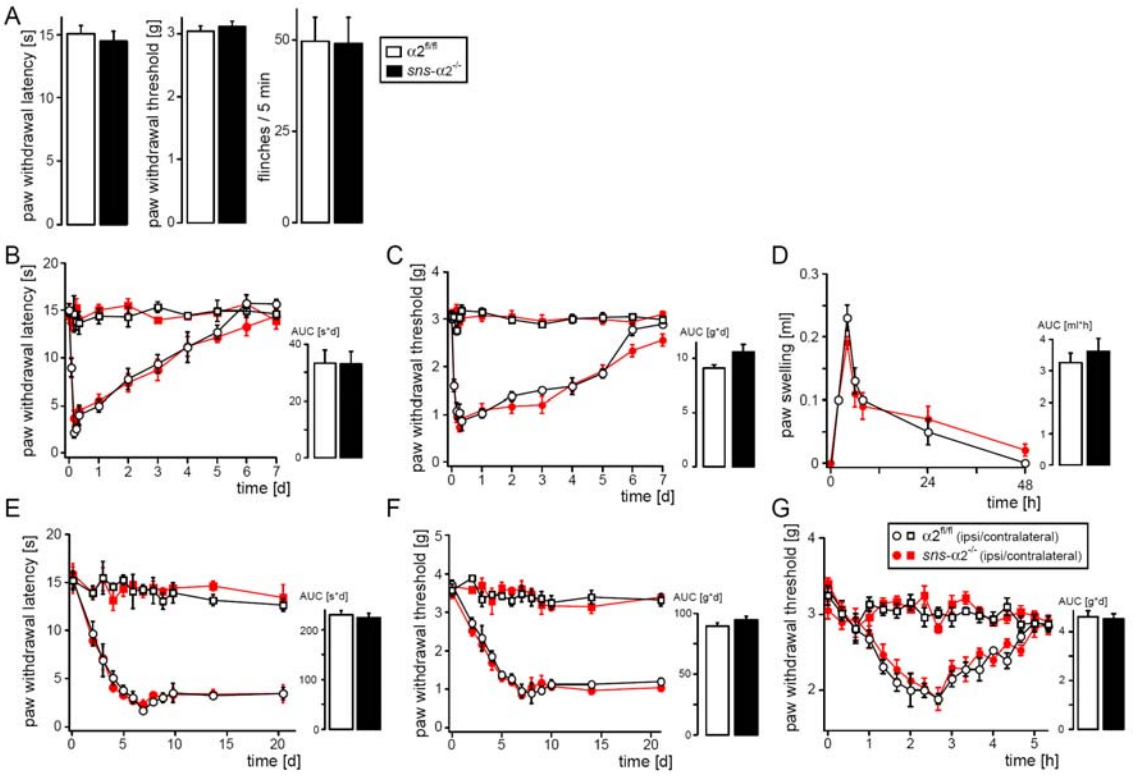


Figure 5. **Nociceptive behavior in *sns*-α2$^{-/-}$ mice.**

(A) Baseline nociceptive sensitivity. Paw withdrawal latencies (s) in response to stimulation with a defined radiant heat stimulus (left), mechanical thresholds (g) to stimulation with dynamic von Frey filaments (middle), and numbers capsaicin-induced flinches (right) in *sns*-α2$^{-/-}$ mice and in wild type (α2$^{fl/fl}$) littermates. Mean ± sem, *n* = 6-10 mice / group, *P* > 0.05 (unpaired *t* test). (B-D) Inflammation induced by subcutaneous zymosan A injection (0.06 mg/ 10 µl) into the plantar side of the left hind paw. Thermal hyperalgesia (paw withdrawal latencies, s) (B), mechanical sensitization (withdrawal thresholds, g) (C), and paw swelling (D) in *sns*-α2$^{-/-}$ and wild type (α2$^{fl/fl}$) mice. Left: time course, right: statistics, area under the curve (AUC, mean ± sem). *n* = 6 - 10 mice / group, *P* > 0.05 (unpaired *t* test). (E, F) Same as B, C, but neuropathic pain induced through chronic constriction injury of the left sciatic nerve. *n* = 6 mice / group. (G) Secondary hyperalgesia induced through subcutaneous injection of capsaicin (30 µg / 10 µl) into the plantar left hind paw. Mechanical withdrawal thresholds (g). Left: time course, right: statistics, area under the curve (AUC, mean ± sem). Mean ± sem, *n* = 5 - 6 mice/group. *P* > 0.05 (unpaired *t* test).
(A-G) open symbols, wild type (α2$^{fl/fl}$); filled symbols, *sns*-α2$^{-/-}$; (B-G) circles, ipsilateral paws; squares, contralateral paws.

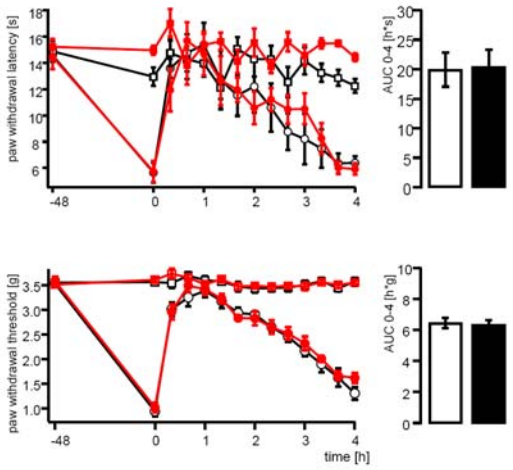


Figure 6. **Antihyperalgesic effects of dzp in *sns-cre*$^{tg-}$ and *sns-cre*$^{tg+}$ mice in the Zymosan A inflammatory model.**

Antihyperalgesic effects of intrathecal dzp (0.09 mg / kg body weight) on thermal (top), and mechanical (bottom) hyperalgesia expressed as paw withdrawal latency (s) or paw withdrawal threshold (g), respectively. AUC, area under the curve (0 - 4 h after dzp injection).
Inflammatory hyperalgesia induced by subcutaneous zymosan A injection (0.06 mg / 10 μl) into the left hind paw. Dzp was given 48 hours after zymosan A injection.
Mean ± sem, *n* = 7-9 mice/group. *P* > 0.05 (unpaired *t*-test). Open black symbols, (*sns-cre*$^{tg-}$); filled red symbols, *sns-cre*$^{tg+}$; circles, ipsilateral paws; squares, contralateral paws.

In a separate set of experiments we assessed the consequences of the *sns*-α2 gene deletion for the antihyperalgesic effect of spinally applied dzp in the three pathological pain models. The effect of dzp (0.09 mg/kg body weight, compare (11)) injected intrathecally at the level of the lower lumbar spine was tested when hyperalgesia had reached a maximum, i.e. after 2 and 7 days in the zymosan A and CCI model, respectively, and after 2 hours in the capsaicin model of central hyperalgesia. Dzp reversibly reduced inflammatory thermal hyperalgesia and mechanical sensitization in $\alpha2^{fl/fl}$ mice. This antihyperalgesia was significantly reduced in both *sns*-$\alpha2^{-/-}$ and global α2-$GABA_A$ point mutated mice ($\alpha2^{R/R}$ mice) (Fig. 7A,B). In the experiments addressing mechanical hyperalgesia we included in addition to *sns*-$\alpha2^{-/-}$ and $\alpha2^{R/R}$ mice also nociceptor-specific α2-$GABA_A$ receptor point-mutated mice (*sns*-$\alpha2^{R/-}$), which carry dzp-insensitive α2-$GABA_A$ receptors specifically in primary nociceptors and a global heterozygous point mutation which is not dzp-insensitive in other cells (see figure 1). These mice exhibited a reduction in dzp-induced antihyperalgesia, which was similar that observed in *sns*-$\alpha2^{-/-}$ rendering compensatory up-regulations of other dzp-sensitive $GABA_A$ receptors in the *sns*-$\alpha2^{R/-}$ unlikely. Importantly, *sns*-$\alpha2^{+/-}$ (hemizygous nociceptor-specific knock-out) mice, and $\alpha2^{R/fl}$ (heterozygous point mutated) mice did not differ significantly from $\alpha2^{fl/fl}$ mice (data not shown).

Although intrathecal dzp was similarly effective in inflammatory and neuropathic pain states and although both effects were mainly mediated by α2-$GABA_A$, neither *sns*-$\alpha2^{-/-}$ nor *sns*-$\alpha2^{R/-}$

mice exhibited reduced dzp-induced antihyperalgesia in the CCI model of neuropathic pain (Fig. 7C,D) and in capsaicin-induced central hyperalgesia (Fig. 7E).

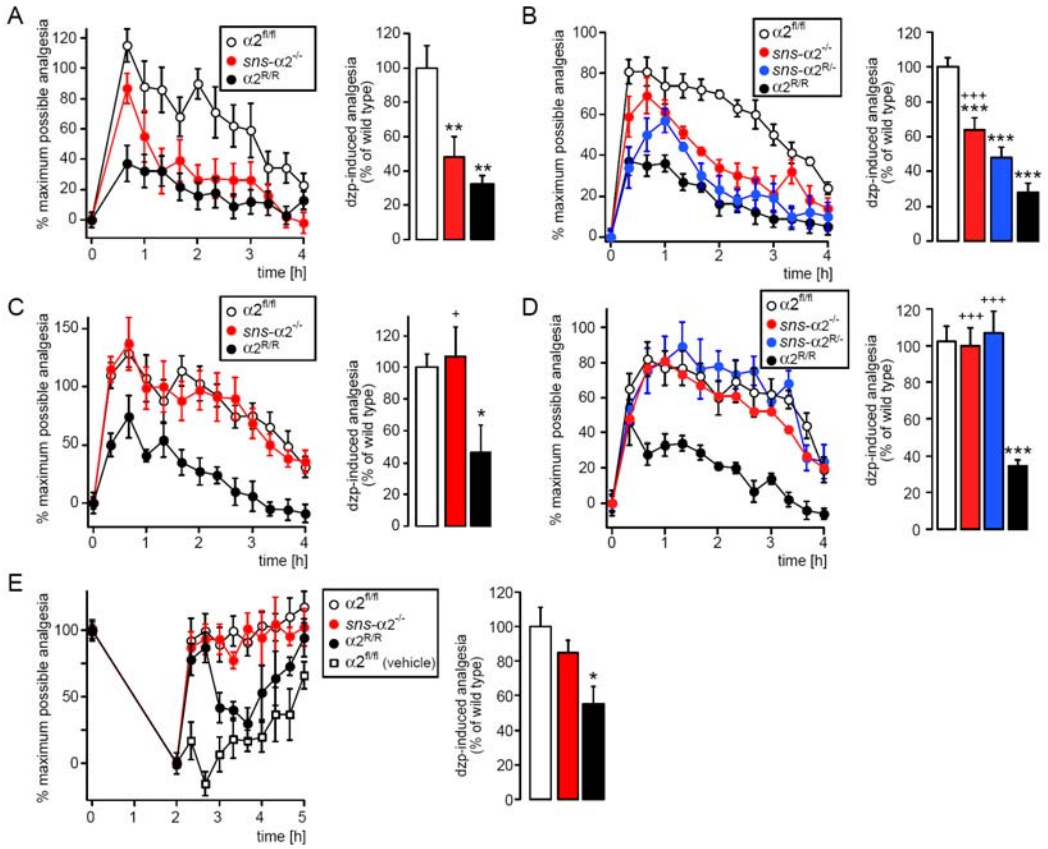


Figure 7. **Anti-hyperalgesic effects of dzp in *sns*-α2$^{-/-}$ and *sns*-α2$^{R/-}$ mice.**

Anti-hyperalgesic effects of intrathecal dzp (0.09 mg / kg body weight) on thermal (A,C), and mechanical (B,D,E) hyperalgesia expressed as per cent maximum possible analgesia (mean ± sem). AUC, area under the curve (0 - 4 h after dzp injection). (A, B) Inflammatory hyperalgesia induced by subcutaneous zymosan A injection (0.06 mg / 10 μl) into the left hind paw. Dzp was given 48 hours after zymosan A injection Left: time course; Right: statistics. AUC expressed as per cent of wild type littermates (α2$^{fl/fl}$ mice). ANOVA F (2,25) = 8.71 followed by Bonferroni post hoc test, n = 8 - 10 mice / group (thermal hyperalgesia); ANOVA F (3,33) = 36.82, n = 7 - 12 mice/group (mechanical hyperalgesia). (C, D) Same as (A, B) but neuropathic pain induced by chronic constriction injury of the left sciatic nerve. Anti-hyperalgesic effects were assessed 7 days after surgery. Statistics: ANOVA followed by Bonferroni post hoc test F (2,21) = 5.18, n = 7 - 9 mice / group (thermal hyperalgesia); F (3,23) = 11.16, n = 5 - 10 mice/group (mechanical hyperalgesia). (E) Same as B, but secondary hyperalgesia induced through subcutaneous injection of capsaicin (30 μg / 10 μl) into the plantar left hind paw. Dzp was injected 2 hours after capsaicin. AUC, 0 – 3 h after dzp injection. Statistics: ANOVA followed by Bonferroni post hoc test F (3,31) = 17.15, n = 6 - 12 mice / group. *, **, *** $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, significant against α2$^{fl/fl}$, +, +++; $P \leq 0.05$, $P \leq 0.001$, against α2$^{R/R}$.

Discussion

We have used here a genetic approach to investigate the contribution of presynaptic $GABA_A$ receptors on the spinal nociceptor terminals to pain control under physiological conditions and in different pain pathologies. Previous *in-situ* hybridization (34, 35), immunofluorescence (1, 11), and electrophysiological (11) experiments have suggested that presynaptic $GABA_A$ receptors on dorsal horn nociceptor terminals are mainly, if not exclusively, of the $\alpha 2$ subtype. Our confocal double labeling experiments indeed show that a large portion (between one third and one forth) of the peptidergic and non-peptidergic fibers express $\alpha 2$-$GABA_A$ receptors, but they also carry comparable amounts of $\alpha 3$ subunits. This is consistent with the results of our electrophysiological experiments, which demonstrated the presence of GABAergic membrane currents of similar amplitude in nociceptive DRG neurons from *sns*-$\alpha 2^{-/-}$ mice. The diminished dzp-sensitivity of these currents may suggest an additional compensation by dzp-insensitive GABA receptors formed e.g. by ρ subunits which have previously been reported in nociceptive DRG neurons (36).

The most obvious phenotype observed in the *sns*-$\alpha 2^{-/-}$ mice was a reduction in the antihyperalgesic effect of intrathecal dzp against inflammatory hyperalgesia showing that a significant part of this antihyperalgesic effect originated from increased presynaptic GABAergic inhibition. The total contribution of the $\alpha 2$ subunit to spinal dzp-mediated antihyperalgesia was about 70%. Of these 70%, about two thirds were lost both in nociceptor-specific knock-out (*sns*-$\alpha 2^{-/-}$) and knock-in (*sns*-$\alpha 2^{R/-}$) mice. This presynaptic component was similar for both thermal and mechanical hyperalgesia indicating that the responses to both types of stimuli were triggered by nociceptors, and that large myelinated fibers were less important. The reduced antihyperalgesia in *sns*-$\alpha 2^{-/-}$ mice correlates well with our electrophysiological data which indicated a reduced dzp-sensitivity of GABAergic membrane currents in primary nociceptors and a diminished ability of dzp to increase the failure rate of primary afferent evoked EPSCs recorded from superficial dorsal horn neurons.

The $\alpha 2$-$GABA_A$ receptor-mediated antihyperalgesia remaining in *sns*-$\alpha 2^{-/-}$ mice most likely originates from postsynaptic $\alpha 2$-$GABA_A$ receptors, whose existence in the dorsal horn has not been generally accepted previously, because *in situ* hybridization studies had revealed significant amounts of $\alpha 2$ mRNA only in the ventral and not in the dorsal horn (34). However, the present immunofluorescence experiments, in which most of the $\alpha 2$ fluorescence in the superficial layers was retained in *sns*-$\alpha 2^{-/-}$ mice, and our previous electrophysiological experiments, which had shown a reduction in dzp-sensitivity of dorsal horn neurons of $\alpha 2^{R/R}$ mice (11), strongly support a significant postsynaptic localization also in the dorsal horn.

In striking contrast with the antihyperalgesic activity of spinal dzp against inflammatory pain, its activity against neuropathic pain induced by sciatic nerve constriction was not changed in

sns-$\alpha2^{-/-}$ nor *sns*-$\alpha2^{R/-}$ mice. This is particularly surprising because the total contribution of α2-$GABA_A$ receptors to dzp-induced spinal antihyperalgesia was very similar in both pain models. Two findings from other groups may offer an explanation. Early studies on dorsal root potentials in rats demonstrated reduced GABA sensitivity of spinal primary afferent nerve terminals after peripheral nerve injury (37). More recently, gene expression studies in DRGs revealed a reduced expression of the $GABA_A$ receptor γ2 subunit (38) which is required for dzp-induced potentiation of $GABA_A$ receptor activation (but see also (39)).

The fact that nerve injured *sns*-$\alpha2^{-/-}$ or *sns*-$\alpha2^{R/-}$ mice responded normally to intrathecal dzp indicates that postsynaptic α2-$GABA_A$ receptors play a dominant role in antihyperalgesia against nerve injury-induced neuropathic pain. This is important because Coull et al. (4, 5) have provided data indicating a depolarizing shift in the neuronal chloride gradient potentially turning GABAergic inhibition into excitation in the course of CCI neuropathy. The present data suggest that dorsal horn $GABA_A$ receptors retain an inhibitory action even after peripheral nerve injury.

$GABA_A$ receptors on spinal nociceptor terminals have been suggested to inhibit the transmission of nociceptive signals through primary afferent depolarization and resulting presynaptic inhibition. The *sns*-$\alpha2^{-/-}$ mice studied here had normal baseline nociceptive sensitivity and developed normal inflammatory or neuropathic hyperalgesia. Under conditions of very intense nociceptor stimulation or when inflammation-induced changes in the expression of chloride transporters further enhance PAD to potentials sufficient of triggering action potentials, PAD may on the contrary also exaggerate pain and inflammation through so called dorsal root reflexes (28, 40). Under these conditions, input to the dorsal horn from one primary afferent nerve fiber could via an interconnected GABAergic interneuron activate another primary afferent fiber terminal. In this fiber, the action potential could then propagate both anterogradely into the synaptic terminal, and retrogradely along the nerve into the peripheral tissue, where it could in case of a peptidergic fibers release proinflammatory mediators such as CGRP and substance P. Again, *sns*-$\alpha2^{-/-}$ mice did not exhibit altered hyperalgesia after capsaicin injection and no changes in hyperalgesia or paw swelling after capsaicin or zymosan A injection. However, both of these findings do not exclude a contribution of PAD to either presynaptic inhibition or dorsal root reflexes, because the $GABA_A$ receptors remaining in nociceptors of *sns*-$\alpha2^{-/-}$ mice were apparently sufficient to sustain GABAergic membrane currents of normal amplitude.

It has also been speculated that the antihyperalgesic action spinal benzodiazepines might be limited by a possible facilitation of $GABA_A$ receptor-mediated dorsal root reflexes. However, although GABAergic membrane currents in nociceptive DRG neurons of *sns*-$\alpha2^{-/-}$ mice were less dzp sensitive, we did not observe increased antihyperalgesic effects in any of the pain models employed here.

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AUTOR CONTRIBUTIONS

R.W. performed all behavioral experiments and made the qRT-PCR experiments, U.R. and R.K. generated floxed *gaba2* mice, P.P. did the electrophysiological experiments, J.P. and J.M.F. made all morphological analyses, H.U.Z. designed the experiments, R.W. and H.U.Z. wrote the manuscript, all authors made comments to the manuscript.

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3.3 Hoxb8-Cre Mice: a Tool for Brain-Sparing Conditional Gene Deletion*

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Abstract

The spinal cord is the first site of the temporal and spatial integration of nociceptive signals in the pain pathway. Neuroplastic changes occurring at this site contribute critically to various chronic pain syndromes. Gene targeting in mice has generated important insights into these processes. However, the analysis of global gene-deficient mice is often hampered by confounding effects arising from supraspinal sites. Here, we describe a novel *Homeobox-8-Cre* (*Hoxb8-Cre)* mouse line which expresses the *Cre* recombinase under the transcriptional control of the *Hoxb8* gene. Within the neural axis of the mice, *Hoxb8-Cre* expression is found in spinal cord neurons and glial cells, and in virtually all neurons of the dorsal root ganglia, but spares the brain apart from few cells in the spinal trigeminal nucleus. The *Hoxb8-Cre* mouse line should be a valuable new tool for the *in vivo* analysis of peripheral and spinal aspects of pain.

Introduction

Noxious (i.e. painful or potentially tissue damaging) stimuli are sensed by specialized nerve cells, called peripheral or primary nociceptors, which connect the peripheral tissues with the spinal cord dorsal horn, the first site of synaptic processing in the pain pathway. From there, nociceptive signals are relayed to higher central nervous system areas where pain finally becomes conscious. It is generally accepted that chronic/pathological pain syndromes can originate from dysfunctions at all three levels. Persistent activity of peripheral nociceptors as well as plastic changes in the spinal and supra-spinal processing of nociceptive stimuli have been shown to contribute to these pathologies. In addition, these sites are also critically involved in the action of many analgesic drugs, in particular of opioids (Dickenson & Kieffer, 2006) but also of aspirin-like drugs (cyclooxygenase inhibitors) (Malmberg & Yaksh, 1992a; Malmberg & Yaksh, 1992b). Conventional (global) gene targeting has yielded important insights into mechanisms of pain and analgesia. It does however not allow discrimination between the different sites, although this was highly desirable in many aspects of basic pain research and analgesic drug development. One strategy to address this issue relies on conditional gene deletion through the *Cre-lox*P system. Primary nociceptor-specific gene deletion can be achieved with mice expressing the *Cre* recombinase under the transcriptional control of the gene encoding the sensory neuron-specific sodium channel (*sns*) *Nav1.8* (Agarwal et al., 2004; Akopian et al., 1999). Other mouse lines, *Peripherin-Cre* (Zhou et al., 2002) and *HtPA-Cre* (Pietri et al., 2003), have been reported to express the *Cre* recombinase in primary sensory neurons of dorsal root ganglia. To further discriminate spinal (and peripheral) sites from supraspinal sites, we aimed at the generation of a *Cre* mouse line allowing brain-sparing gene deletion. To this end, we generated a novel mouse line expressing the *Cre* recombinase under the transcriptional control of the murine homeobox

gene *Hoxb8 (Hox-2.4)*. *Hox* genes are expressed in spatially and temporally restricted domains along the anterior-posterior axis of the body, where they usually show a sharp rostral expression boundary . The expression of of the *Hoxb8* gene extends to the cervical segment C2 (Charité et al., 1995; Deschamps and Wijgerde, 1993). The expression pattern described above makes *Hoxb8* an appropriate gene to drive *Cre* expression for brain-sparing gene deletion.

Materials and Methods

Generation of Transgenic Construct and Mice. The *Hoxb8-lacZ* reporter construct number 1 (kindly provided by Jacqueline Deschamps (ref.)) was modified by restriction enzymes. The 11,410 bp genomic regions 5' and 3' between *Sal I* (*Hoxb9*, exon1) and ~20 bp upstream to the *Nru I* site (within *lacZ*) from the *Hoxb8-lacZ* reporter construct, containing the first 1058 bp of the *Hoxb8* sequence (whole genomic sequence before ATG starting codon of Hoxb8), was fused to a sequence containing a Kozak sequence and ATG starting codon (5'-ACGCCACCATG-3') into a cloning vector (pBC SK (+/-), Stratagene). A *Cre* recombinase construct containing a bovine poly (A) sequence was cloned 24 bp downstream of vector sequence (5'-GGACCCAAGAAGAAGAGGAAGGTG-3') to the ATG starting codon. The final linearized construct (12,754 bp) was sequenced, purified for microinjection and injected into early state oocytes from C57BL/6 x DBA2 mice. Four transgenic founders were obtained which were crossed back to C57BL/6 background for at least 2 generations before crossing these *Hoxb8-Cre* heterozygous mice with the *RA/EG* and *ROSA26lacZ* reporter mouse lines. The analyzed mice had a C57BL/6 background of at least 93.75 %.

Southern Blot Analysis. The number of *Hoxb8-Cre* transgene copies integrated into the genome of *Hoxb8-Cre* mouse line 1403 was determined using quantitative southern blot. A probe (749 bp) was generated by PCR using the following primers: FWD: 5'-TTG TTG TGA GGC AAG AGA TA-3' and REV: 5'-TTT ATT GAA TTT TGA GGC G-3' labelling *Hoxb8* promoter region. *EcoRV* digestion of genomic liver DNA resulted in a 4,4 kb wild type and transgenic 1,3 kb *Hoxb8-Cre* band.

LacZ Staining and Immunohistochemistry. To study the pattern of *Cre* activity the mouse lines were crossed with mice from 2 reporter mouse lines, B6.129S4-Gt(ROSA)26Sortm1Sor/J (*ROSA26lacZ*) (Soriano, 1999) and B6.129P2-Ager ^{tm1Arnd} (*RA/EG*) Constien et al., 2001), as well as with the use of mice carrying a floxed GlyT1 allele (GlyT1$^{fl/fl}$; Slc6a9$^{tm1.1Bois}$). Co-transgenic progeny (4-6 weeks) from Hoxb8-Cre and ROSA26-lacZ mice were histologically analyzed for β-galactosidase activity in cryostat-sections (16 μm) of DRGs, spinal cord, brain, liver, heart and kidney (for protocol see Hogan et al., 1994).

Counterstaining was performed with acidified (4% acetic acid) hematoxylin. Whole-mount embryos (plug was considered as embryonic day E 0.5) were freed of their extraembryonic membranes before being fixed in 0.2% glutaraldehyde between 15 and 30 min on ice. *LacZ* staining was performed at 37°C for 1-24 hours (for protocol see Hogan et al., 1994).

For immunostaining experiments, mice (age 4-6 weeks, GlyT1 experiments: 10 days) were perfused transcardially through the ascending aorta with PBS followed by fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.15 M phosphate buffer, pH7.4. After perfusion, the organs were dissected rapidly and postfixed over night in the same fixative solution followed by cryoprotection in PBS containing 30% sucrose over night (until tissue blocks sinked). The organs were cut at -40°C into 40 µm sections with a sliding microtome HM400 (Micron, Heidelberg). The free-floating sections were washed in Tris-Triton (Tris-buffer with 0.05% Triton X-100, pH 7.4) and then incubated in a mixture of primary antibodies diluted in Tris-Triton containing 2% normal goat serum (NGS) and 0.2% Triton X-100 in a moist chamber with continuous agitation (100 rpm) overnight at 4°C. Sections were then washed 3 times 10 min in Tris-Triton and incubated for 30 min at room temperature in a mixture of secondary antibodies coupled to the fluorochromes Alexa Fluor488 and cyanine dye Cy3 (Jackson Immunoresearch, West Grove, PA). Non specific staining was blocked with 2% normal goat serum. Sections were washed 3 times 10 min in PBS and mounted on gelatine-coated slides, air dried and coverslipped with fluorescence mounting medium (Dako Cytomation). Antisera used: Chicken bacterial anti-β-galactosidase 1:3000 (Abcam, ab9361), NeuN 1:5000 (Chemicon), rabbit anti-GlyT1a,b 1:1000 (same as being used in the publication Zeilhofer et al., 2005). The lacZ stainings and immunostainings were analyzed using the Axioskop 2 MOT (Carl Zeiss AG), the Axiocam color (Carl Zeiss AG), Axioplan 2 (Carl Zeiss AG) and the laser scanning confocal microscope LSM 510 Meta (Carl Zeiss AG). Image acquisition parameters were adjusted to the full dynamic range of the photodetector and all images from a particular experiment were taken with the same settings. Confocal images were processed with the image analysis software Mac Biophotonics ImageJ.

Testing of Paw Withdrawal Reflexes. Behavioral measurements were done on awake, free-moving mice (age 9 weeks). The mouse plantar test apparatus (Ugo basile, Italy) was used to determine paw withdrawal latencies in response to noxious heat, which was applied via an infrared light source. Similarly, dynamic von Frey filaments (IITC) were used to apply increasing mechanical pressure to the plantar surface of one hindpaw and mechanical stimulus thresholds were recorded in grams. At least 5 measurements were recorded per paw and animal. (n = 4-6 mice per group).

Animal permission for all animal experiments was obtained from the Veterinäramt of the Kanton of Zurich (licences 34/2007 and 35/2009).

Data and Statistics. Numeric data are presented as mean +/- SEM. In figure 5, the *Hoxb8-Cre* and littermate wild type groups were compared with unpaired Student *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Charité et al. characterized upstream cis-acting regulatory elements of the *Hoxb8* gene and found that a 11kb DNA segment upstream of the *Hoxb8* translational start closely mimicked the endogenous *Hoxb8* expression pattern. To generate *Hoxb8-Cre* transgenic mouse lines, we fused the 11 kb DNA segment to a *Cre* expression cassette and used this construct for pronuclear injection (figure 1A). Four transgenic founders were obtained and gave rise to four transgenic lines, which were back crossed continuously to the C57BL/6 background and maintained in a heterozygous state. Two lines (1403, 1404) showed the desired expression pattern on a gross scale (figure 1B). One of these lines (1403), which carries a single copy of the transgene (figure 1C), is described here in detail.

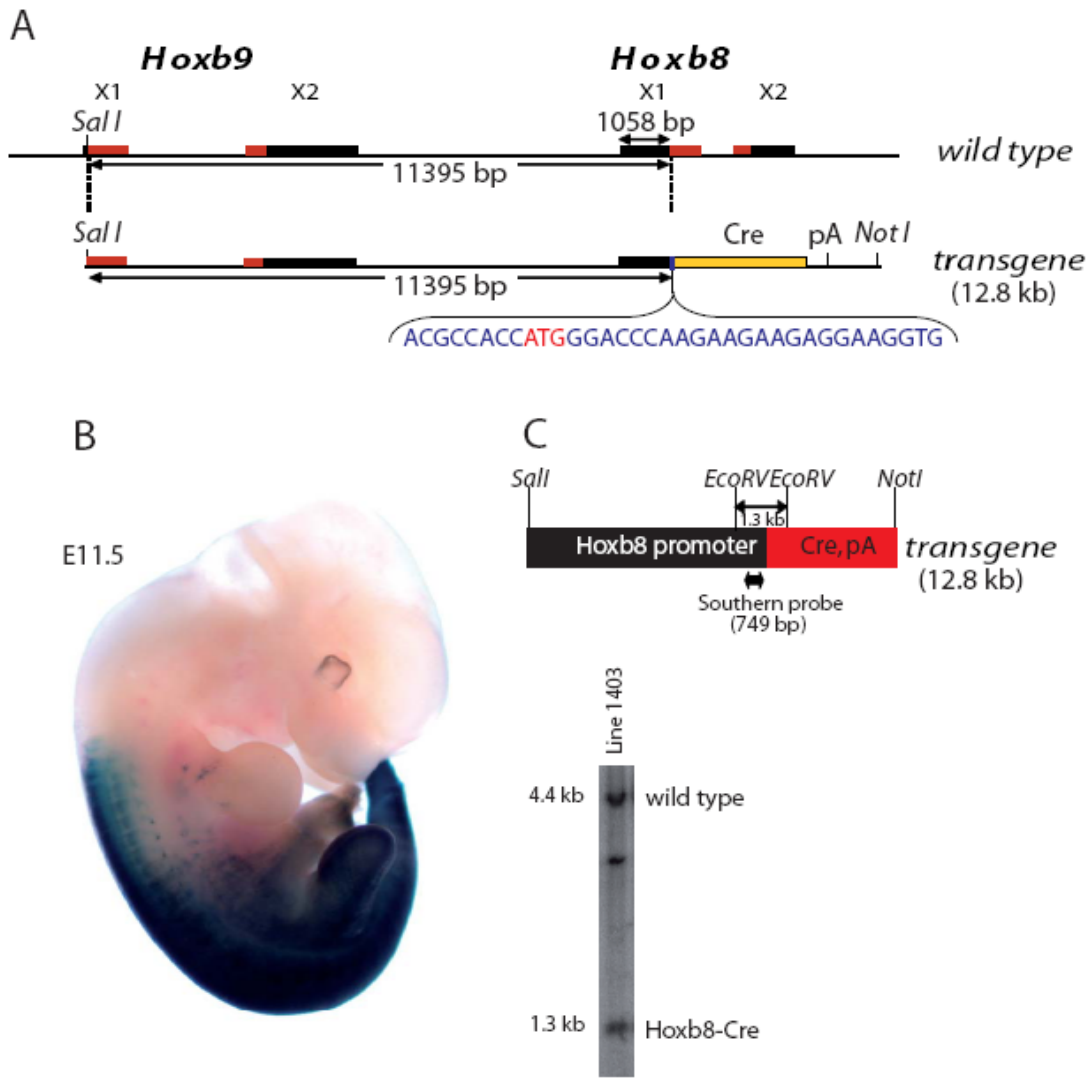

Figure 1. **Generation of *Hoxb8-Cre* mice.**

(A) Cloning construct and respective genomic context in the murine locus. Red bars indicate coding regions of exons (X). Between *Hoxb9*- and *Hoxb8* genes an artificial sequence (blue letters) containing a starting ATG codon was inserted. **(B)** *Hoxb8-Cre*-induced *lacZ* activity in E11.5 embryo. **(C)** Southern blot of *EcoRV* digested genomic liver DNA from a *Hoxb8-Cre* transgenic mouse (line 1403) hybridized with a probe against the *Hoxb8* promoter. A hybridization intensity ratio of about 0.6 between *Hoxb8-Cre* transgene (1.3 kb) and *Hoxb8* wild type (4.4 kb) suggests the presence of a single copy of the transgene.

To analyze the expression pattern of *Hoxb8-Cre*-mediated gene recombination along the neural axis, *Hoxb8-Cre* mice were crossed with *Rosa26lacZ* mice (*R26R*) . Cryostat sections from spinal cords, brains and spinal dorsal root ganglion (DRG) neurons (which harbour the somata of peripheral sensory neurons, including nociceptors) were prepared from adult co-transgenic (*Hoxb8-Cre^{tg+}/R26R)* mice and stained with X-Gal followed by a counterstain with acidified hematoxylin. Sagittal and coronal spinal cord sections from adult co-transgenic mice revealed a *lacZ* expression pattern throughout the white and grey matter of the spinal cord in a pattern reminiscent of a Nissl staining, suggesting *lacZ* expression in neurons as well as in glial cells (figure 2A). Wild type littermate control sections which were included as controls did not show any visible *lacZ* activity. To determine the rostral *Cre* expression boundary in the *Hoxb8-Cre* mouse line, coronal and horizontal spinal cord sections representing different anterior-posterior spinal cord segments were analyzed. *Hoxb8-Cre*-induced *lacZ* expression was similar at the lumbar and thoraxic segment, but gradually decreased in a caudo-rostral direction within the cervical segment (figure 2C). While full *lacZ* activity was still observed at the cervical segment C7 in both the grey and the white matter, *lacZ* activity disappeared around the cervical segment C4 and became restricted to a few cells scattered in the grey matter at cervical segment C2. The brain was largely devoid of *lacZ* precipitates (figure 2D) even after prolonged (24 hours) X-Gal exposure with the exception of a few cells in the spinal trigeminal nucleus (figure 2E).

Hoxb8-Cre-mediated gene recombination was also analyzed in cryostat sections of lumbar DRGs from *Hoxb8-Cre^{tg+}/R26R* mice that showed *Cre*-induced *lacZ* activity in virtually all cell bodies of both small and large DRG neurons, indicating efficient *Cre*-mediated gene recombination in all cell bodies of primary afferent neurons, including primary nociceptors (Figure 2B).

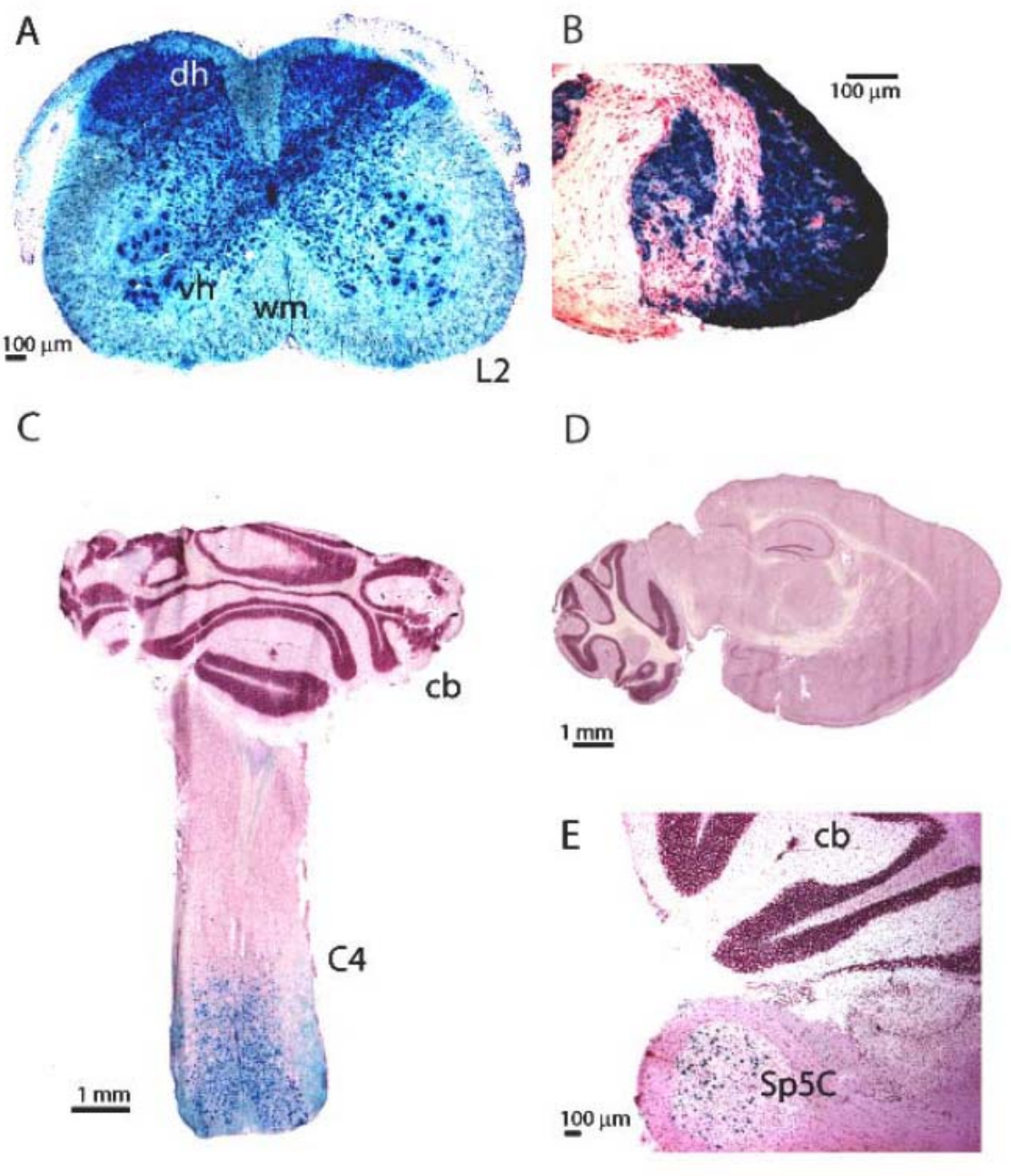

Figure 2. **β-gal activity in co-transgenic *Hoxb8-Cre*$^{tg+}$*/R26R* mice in neural tissue**.

Counterstaining with acidified hematoxylin. **(A)** Coronal section of the spinal cord at lumbar segment L2. Dorsal horn (dh); ventral horn (vh); white matter (wm). **(B)** Lumbar dorsal root ganglion. **(C)** Horizontal section of the upper cervical spinal cord and cerebellum (cb) showing a gradual decrease of β-gal activity towards more anterior cervical segments. **(D)** Sagittal brain section showing no visible *Hoxb8-Cre*-induced β-gal activity **(E)** Sagittal section including brainstem, spinal trigeminal nucleus (Sp5C), and cerebellum (cb).

We next aimed at determining the types of cells showing *Hoxb8-Cre*-induced *lacZ* precipitates in the spinal cord. To demonstrate the presence of *lacZ* in neurons, we performed co-immunostainings of coronal spinal cord sections with anti-sera against the bacterial β-galactosidase (β–gal) and the neuron-specific nuclear protein (NeuN). β–gal immunoreactivity was found in virtually all NeuN-positive neurons (figure 3a,b).

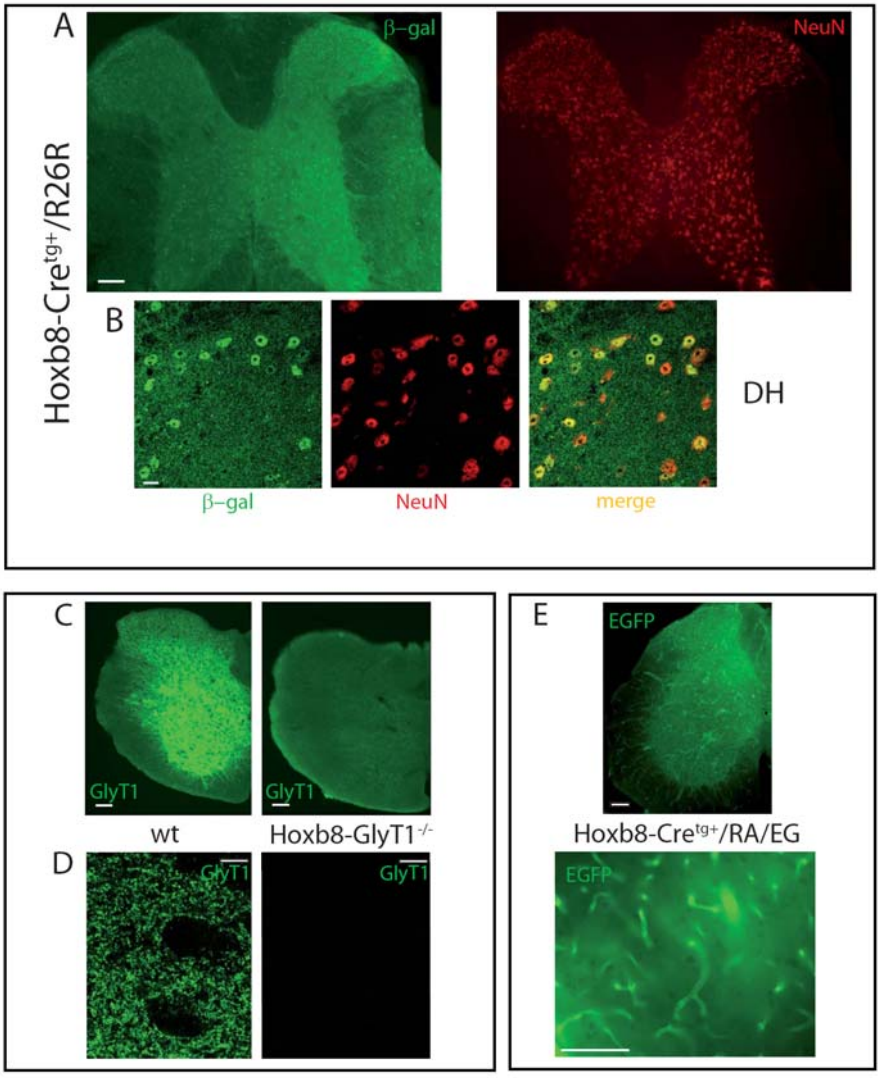

Figure 3. **Histochemical analysis of co-transgenic progeny of *Hoxb8-Cre* mice crossed with *R26R* and *RA/EG* reporter strains**.

Neuronal expression analysis (A,B).
(A) Coronal thoraxic spinal cord section from a co-transgenic *Hoxb8-Cre^{tg+}/R26R* mouse. β–gal (Alexa Fluor488; left panel) and NeuN (Cy3; right panel) immunofluorescence on the same section. Scale bar: 100 μm.
(B) Confocal immunofluorescence analysis of a coronal lumbar spinal cord section from a co-transgenic *Hoxb8-Cre^{tg+}/R26R* mouse. β-gal (Alexa Fluor488), NeuN (Cy3) and merged view in the dorsal horn (DH). Scale bar: 20 μm.
Glial expression analysis (C, D). (C) Coronal section from the lumbar spinal cord of *Hoxb8-GlyT1$^{-/-}$* and wild type littermate mice showing GlyT1 immunofluorescence (Alexa Fluor488). Scale bar: 100 μm. **(D)** Same as (C), but higher magnification and confocal analysis (Alexa Fluor488). Scale bar: 5 μm.
Mesodermic expression analysis (E). Coronal spinal cord sections from a *Hoxb8-Cre^{tg+}/RA/EG* mouse showing strong EGFP signals in lumbar (L3) spinal sections. No EGFP signals were detectable in higher cervical segments (data not shown). Scale bar: 100 μm.

Hoxb8-Cre-mediated recombination could also be verified in astrocytes. This was shown through the *Hoxb8-Cre*-mediated (conditional) deletion of glycine transporter type 1 (GlyT1) gene, which is abundantly expressed in spinal glial cells (Zafra et al., 1995). *Hoxb8-Cre* mice were crossed with mice carrying floxed GlyT1 alleles (Yee et al, 2006) to generate *Hoxb8-Cre^{tg+}/GlytT1$^{flox/flox}$* (*Hoxb8-GlyT1$^{-/-}$*) mice. Immunohistochemical analysis of postnatal day 10 (P10) mice revealed intense GlyT1 immunofluorescence throughout the spinal grey matter of wild type mice, but not in *Hoxb8 -GlyT1$^{-/-}$* mice (figure 3 C,D).

The mesodermic expression pattern in the spinal cord was analyzed with the use of the *RA/EG* reporter strain , which carries a *Cre*-inducible enhanced green fluorescence protein (EGFP) reporter gene in the locus of the receptor for advance glycated end products (*RA/EG*). We analyzed coronal sections at different spinal cord segments of co-trangenic progeny from *Hoxb8-Cre* mice crossed with the *RA/EG* strain (figure 3 E). At lumbar segments, strong *Hoxb8-Cre*-induced EGFP fluorescence was found in cell layers along blood vessels. EGFP fluorescence extended rostrally up to thoraxic segments (approx. T2; data not shown). *RA/EG* reporter mice did not reveal neural *Hoxb8-Cre*-mediated EGFP fluorescence, consistent with previous findings showing that the RA/EG promoter is not constitutively active in most neurons (Brett et al., 1993; Constien et al., 2001).

We next analyzed *Hoxb8-Cre*-induced *lacZ* expression in non-neural tissues. Heart and liver sections did not reveal any apparent *Hoxb8-Cre*-induced *lacZ* staining, whereas kidney sections showed strong *lacZ* activity in about 50% of epithelial cells and in cells suurounding blood vessels (figure 4).

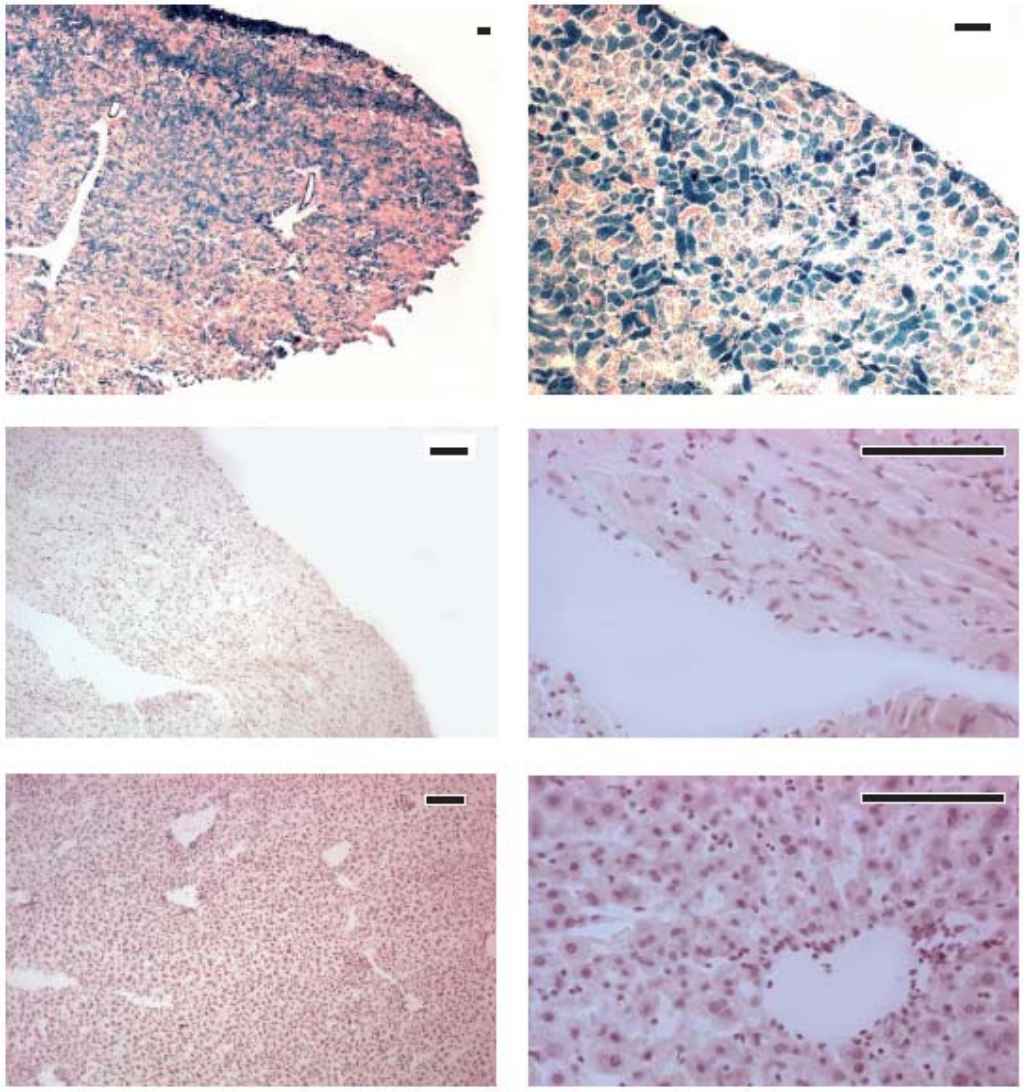

Figure 4. **β-gal activity in co-transgenic *Hoxb8-Cre^{tg+}/R26R* mice in non-neural tissue.**

Histological analysis of β–gal activity in 5-6 week old co-transgenic *Hoxb8-Cre^{tg+}/R26R* progeny. Counterstain with acidified hematoxylin. Kidney (upper row), heart (middle row), liver (lower row). Scale bars represent 100 μm.

Finally, the temporal onset of *Hoxb8*-induced *Cre* activity was analyzed in co-transgenic embryos recovered at embryonic states E9.5 to E15 from *Hoxb8-Cre* mice crossed with the *R26R* strain. A pronounced *lacZ* expression pattern very similar to the rostro-caudal expression pattern observed in adolescent tissues was found already at embryonic day E9.5 (data not shown).

In general, the *Hoxb8-Cre*-induced *lacZ* expression pattern described here is very similar to that of the endogenous *Hoxb8* expression in embryos described earlier (Charité et al., 1995; Deschamps & Wjigerde, 1993). Previous *in situ* hybridization studies localized the

endogenous *Hoxb8* expression boundary at cervical segment C2 (Charité et al., 1995). When neuroectodermal and mesodermic boundaries were compared in an other *in situ* hybridization study, the neuroectodermal *Hoxb8* expression was found to extend more rostrally than the mesodermic expression (6^{th} versus $11/12^{th}$ somite, respectively) (Deschamps & Wijgerde, 1993). Despite these similarities, significant differences exist between the *Hoxb8-Cre*-induced *lacZ* expression in the mice described here and the previously described *Hoxb8lacZ* mice, where *Hoxb8* is replaced by a *lacZ* cassette generated previously . In these *Hoxb8lacZ* mice *lacZ* was more strongly expressed in the dorsal than in the ventral spinal cord and found only in a subpopulation of DRG neurons. By contrast, our transgenic mice expressed *Hoxb8-Cre*-induced *lacZ* similarly in the dorsal and ventral horn of the spinal cord and in all DRG neurons. This and the small difference in the anterior-posterior boundaries of *Hoxb8*-mediated *Cre* expression boundaries (posterior shift of about 2 segments) are most likely due to integration site effects or the absence of regulatory elements of the *Hoxb8* gene, located outside the construct . Since insertion of *Cre* transgenes can potentially lead to a loss of function of genes or to copy-number-dependent *Cre*-induced toxicity (Baba et al., 2005; Silver & Livingston, 2001), one prerequisite for the use of *Hoxb8-Cre* mice in pain studies is that they themselves do not show abnormalities in the responses to painful stimuli. Exposure to noxious thermal or mechanical stimuli did not reveal differences in the nociceptive thresholds of *Hoxb8-Cre* mice compared to their littermate wild type mice (figure 5 A, B).

In summary, the *Hoxb8-Cre* transgenic mice described here showed the desired *Cre* expression pattern and should hence be suitable for brain-sparing gene deletion experiments. This will be very helpful in the site-specific analysis e.g. of pain-related genes which often exhibit a wide-spread expression along the neural axis including peripheral, spinal and supraspinal sites. In such studies, our *Hoxb8-Cre* mice will allow distinguishing effects at supraspinal sites from spinal and peripheral effects.

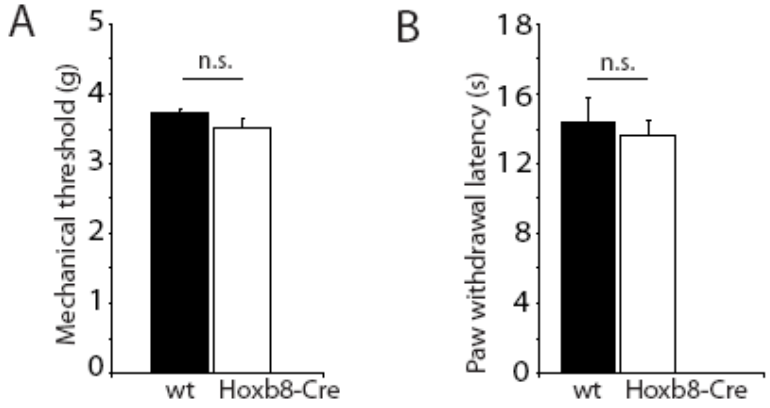

Figure 5. **Responses to noxious thermal and mechanical stimulation in *Hoxb8-Cre* mice.**

Hoxb8-Cre and wild type littermates (wt) show virtually identical mechanical thresholds **(A)** and paw withdrawal latencies upon exposure to noxious heat **(B)** Mean +/- sem. (n = 4-6 mice/group).

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AUTHOR CONTRIBUTIONS

RW performed and analyzed all experiments (except from southern blot analysis) and wrote together with HUZ the manuscript.

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4. Overall Discussion and Future Perspectives

Possible analgesic effects of systemically administered benzodiazepines have been difficult to adress due to their sedative effects. Experiments in $GABA_A$ receptor point-mutated (knock-in) mice, in which specific $GABA_A$ receptor subtypes have been rendered insensitive to diazepam, allowed to assess this question. Genetic and pharmacological evidence gathered in this thesis now clearly indicates that an analgesic effect of benzodiazepines exists after spinal application via benzodiazepine-sensitive $GABA_A$ receptors containing the $\alpha 2$ and/or $\alpha 3$ subunits. Pharmacological tools used subsequently conformed these findings. Systemic application of a non-sedative $\alpha 1$-sparing benzodiazepine site ligand (L-838,417) decreased hyperalgesia in inflammatory and neuropathic pain (paper 1).

Importantly, spinally injected diazepam is not effective against pain in non-inflamed or non-injured animals but only reverses pathologically increased pain sensitivity, a finding that is in good agreement with the notion that benzodiazepines are generally not analgesic (Jasmin et al., 2004). Therefore its action should be considered as anti-hyperalgesic rather than anagesic. The reason for this differential effect is not known. It has been suggested that the GABAergic tone (e.g. descending inhibitory pathways) in the nociceptive system is low under physiological conditions (Dirig and Yaksh, 1995). According to these findings patients suffering from chronic pain associated with a loss of inhibitory neurotransmission in the dorsal horn might benefit from the novel non-sedative site-specific $GABA_A$ receptor ligands.

At present, it remains unknown whether or not these findings can be translated to human patients. Clinical evidence suggests that benzodiazepines lack a clear analgesic effect at low non-sedative doses. The testing of this hypothesis will require the availability of subtype-selective benzodiazepine or $GABA_A$ receptor ligands. Unfortunatley, L-838,417, the compound used in paper 1, possesses undesirable pharmacokinetics in man (Rogawski, 2006). Oher compounds such TPA023 or SL 651498 (de Haas et al., 2007; de Haas et al., 2008) possess suitable pharmacokinetics in man, but have either very low intrinsic activity at $\alpha 2$-$GABA_A$ receptors (Atack et al., 2006) or have a very narrow non-sedative window (Griebel et al., 2001).

Nevertheless, future subtype-selective agonists with suitable pharmacokinetic properties might not only be devoid of sedation, but might also prevent other side effects (liability to physical dependence, addiction and tolerance development) that currently also restrict or even prevent the use of classical benzodiazepines as analgesics. It has already been shown that the amnestic effects of classical benzodiazepines involve $GABA_A$ receptors containing the $\alpha 1$ subunit (Rudolph et al., 2001). Similarly, published evidence suggests that physical

dependence does not occur with several α1-sparing agents (TPA023) (Mirza and Nielsen, 2006) and at least in the case of L-838,417, tolerance against the anti-hyperalgesic effect was completely absent during a 9-day treatment period - in striking contrast to morphine, which had lost its analgesic activity already within 9 days (paper 1). A recent study provided direct evidence that the addictive properties of classicial benzodiazepines depend on α1-containing $GABA_A$ receptors in GABAergic inhibitory neurons in the ventral tegmental area (Tan et al.). These findings indicate that subunit-selective benzodiazepines sparing α1 should be devoid of addiction liability (but see also (Rowlett et al., 2005).

The situation is significantly less clear with respect to cognition-impairing effects of classicial benzodiazepines. Work in mutant mice shows that hippocampus-dependent learning probably involves α5-$GABA_A$ receptors (Crestani et al., 2002), a finding which is supported by the cognition enhancing effects of α5 inverse agonists (Dawson et al., 2006). Experiments in paper 1 suggest that α5-$GABA_A$ receptors may contribute to spinal antihyperalgesic effects under certain conditions (Mirza et al., 2008; Munro et al., 2008).

The pronounced effect of spinally injected diazepam has also interesting neurobiological implications. In the case of the inflammatory pain model, it suggests that the positive modulation of $GABA_A$ receptors can compensate also for the reduction in glycine-mediated neurotransmission that underlies inflammatory hyperalgesia (Ahmadi et al., 2002; Harvey et al., 2004). In the neuropathic pain model it was suggested that a depolarizing shift in the transmembrane chloride gradient of dorsal horn neurons is a possible mechanisms of neuropathic pain (Coull et al., 2003; Coull and Gagnon, 2009). However, the pronounced effect of intrathecally applied diazepam suggests that the net effect of GABA in the spinal dorsal horn remains inhibitory also in neuropathic pain conditions.

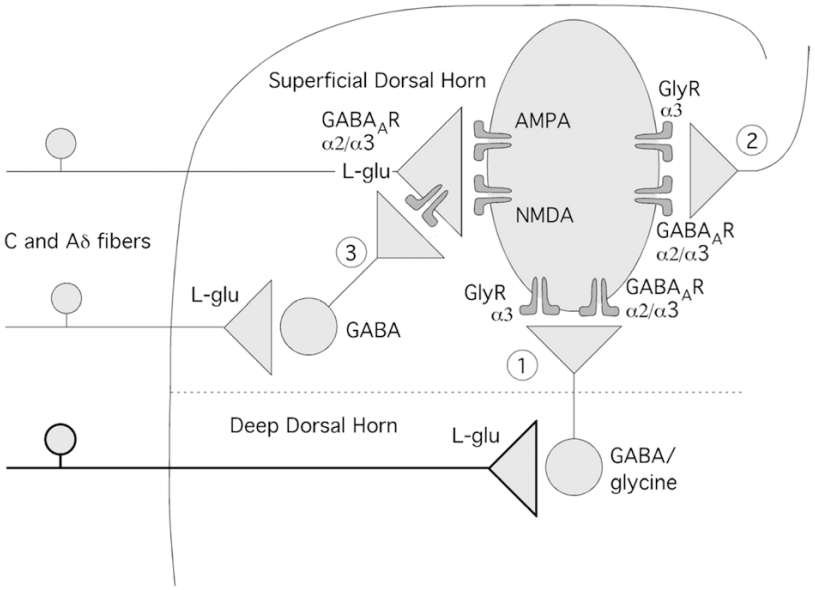
The second project addressed at the contribution of primary afferent depolarization and presynaptic inhibition to spinal benzodiazepine-induced analgesia. It demonstrated that under inflammatory conditions a significant part of the α2-$GABA_A$ receptor-mediated spinal benzodiazepine-induced analgesia originates from $GABA_A$ receptors residing on primary nociceptors. The remaining α2-$GABA_A$ receptor-dependent spinal analgesia could either come from activation of postsynaptically located α2-$GABA_A$ receptors or, theoretically, from α2-$GABA_A$ receptors on myelinated Aβ or Aδ fibers, which were not adressed in this study.

An open question is the possible contribution of supraspinal α2-$GABA_A$ receptors to analgesia evoked by systemically administered benzodiazepines. The *cre* transgenic mouse described in the third project may help to address this question. Here, the generation and characteristics of a new *cre* mouse line (*Hoxb8-cre*) have been described which enables brain-sparing conditional gene deletion in primary sensory neurons and the spinal cord. This mouse line will allow the definition of the contribution of supraspinal α2-$GABA_A$ receptors to GABAergic pain control. Although analgesia induced by intrathecally injected dzp most

probably only involves spinal $GABA_A$ receptors, supraspinal α2-$GABA_A$ receptors might also contribute after systemic administration either directly or through relief from anxiety-induced hyperalgesia. On the other hand, supraspinal $GABA_A$ receptors most likely of the α1 subtype might exert pro-nociceptive actions by increasing the tonic inhibition of antinociceptive fiber tracts descending from the periaqueductal gray (PAG) to the rostral ventromedial medulla (RVM) and the spinal cord. Conditional *Hoxb8-cre*-mediated deletion of the $GABA_A$ receptor α1 subunit will help resolving these questions.

Targeting fast synaptic neurotransmission in the dorsal horn for the treatment of chronic pain might be a novel rational strategy because both inflammatory and neuropathic pain pathologies converge at a loss of inhibitory pain control, which probably accounts for the most symptoms of chronic pain such as allodynia (pain evoked by even slightest cutaneous stimulation). Instead of interfering with signal transduction pathways specific to either inflammatory pain or neuropathic pain, restoring synaptic inhibition in the spinal dorsal horn should be an effective means against a variety of pain syndromes. In addition to $GABA_A$ receptors glycine receptors might be another target. Given the distinct expression of GlyRα3 in the superficial dorsal horn (Harvey et al., 2004), this subunit should be another promising target for such pharmacological interventions (see figure below). Yet, so far no compounds are available which would act as specific positive allosteric modulators of inhibitory glycine receptors (Laube et al., 2002).

In summary, the results from this dissertation contribute to the development of a rational basis for the development of subtype-selective $GABA_A$ receptor ligands for the treatment of chronic pain, which is often refractory to classical analgesics.



Inhibitory synapses in the spinal dorsal horn circuitry allowing fast synaptic inhibitory transmission.

At least three populations of neurons contribute to the synaptic inhibition in the spinal dorsal horn.
(1) GABAergic and glycinergic neurons, which are mainly located in the deeper dorsal horn, are activated by mechano-sensitive Aδ or Aβ fibers. Many of these neurons release both GABA and glycine simultaneously. The dominant glycine receptor isoform at these synapses contains the α3 subunit, while $GABA_A$ receptors at this site probably mainly contain α2 and/or α3 subunits in addition to a β and a γ2 subunit.
(2) GABAergic and glycinergic inhibition also comes from inhibitory fiber tracts descending from the rostral ventromedial medulla.
(3) Inhibitory interneurons located in the superficial dorsal horn probably form mainly axo-axonic synapses with the spinal terminals primary afferent nerve fibers, which express the α2 and α3 subunits. Adapted and rearranged from (Zeilhofer et al., 2009).

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6. Appendices

6.1 Abbreviations

ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
β-gal	β-galactosidase
BOLD	blood oxygen level dependency
cAMP	cyclic adenosine monophosphate
CB1	cannabinoid receptor type1
CCI	chronic constriction injury
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
CNS	central nervous system
COX	cyclooxygenase
cre/loxP	cyclization recombination protein/locus of X-over P1 (bacteriophage1)
CTB	cholera toxin B subunit
Cy3	indocarbocyanin
DMSO	dimethyl sulphoxide
DRG	dorsal root ganglion
DRR	dorsal root reflexes
Dzp/DZP	diazepam
EGFP	enhanced green fluorescent protein
EP2	prostaglandin E2 receptor
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid GABA receptor GABA type A receptor
$GABA_B$	γ-aminobutyric acid GABA receptor GABA type B receptor
GAD65/67	glutamate decarboxylase isoform 65/ isoform 67
gDNA	genomic deoxyribonucleic acid
GlyRα3	Glycine receptor α3 subtype
GlyT1/GlyT2	glycine transporter type 1 / type 2
GPCR	G protein coupled receptor
Hox	homeobox
IASP	International Association for the Study of Pain
IB4	isolectin IB4
i.p.	intraperitoneal
i.t.	intrathecal
KCC2	K^+-Cl^- cotransporter type 2
LTP	long-term potentiation
mPGES-1	microsomal prostaglandin E2 synthase-1
mRNA	messenger ribonucleic acid
NeuN	neuronal nuclei (neuron-specific nuclear protein)
NGF	nerve growth factor
NK1	neurokinin 1
NKCC1	Na^+-K^+-$2Cl^-$ cotransporter type 1
NMDA	*N*-methyl-*D*-aspartic acid
NSAID	non-steroidal anti-inflammatory drug
PAD	primary afferent depolarization
PAG	periaqueductal gray
PAN	primary afferent nociceptor

PGE_2	prostaglandin E2
PKA	protein kinase A
PKC(γ)	protein kinase C (gamma)
PNL	partial nerve ligation
qRT-PCR	quantitative real-time polymerase chain reaction
RA/EG	receptor for advance glycated end products
RVM	rostral ventromedial medulla
SMT	spinomesencephalic tract
SNI	spared nerve injury
SNL	spinal nerve ligation
SP	substance P
SRT	spinoreticular tract
trkB	tyrosine kinase receptor B
TRPV1 (VR1)	transient receptor potential vanilloid type 1
TTX	tetrodotoxin
SD	standard deviation
SEM	standard error of the mean
SNS	sensory neuron-specific sodium channel
VGCC	voltage-gated calcium channel
Veh	vehicle

6.2 Curriculum vitae

Personal Data

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Date of birth:	19.06.1979 in Bern, Switzerland.
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Education

2000	Matura (grammar school leaving exam) at Gymnasium Bern-Neufeld
2000-2002	Basic studies of Pharmaceutical Sciences (today's "Bachelor Studies") (University of Bern)
2002-2003	Stage in pharmacy (Rathaus Apotheke Bern, Dr. Fritz)
2003-2005	Final studies of Pharmaceutical Sciences (today's "Master Studies") (University of Basel), summary exam grade: 5.4 (6 is maximum)
2005	Diploma work: „Development and *in vitro* release of nasal midazolam formulations"(University of Basel)
2005	Swiss federal diplom „Eidg.dipl. Apotheker" (pharmacist), (University of Basel)
2006-2010	PhD studies in Neuropharmacology at the Institute of Pharmacology & Toxicology (ETH & University of Zürich), supervisor Prof. Dr. H.U. Zeilhofer; PhD program in Neuroscience (ZNZ)

Education related experience outside of R&D

Pharmacist in public pharmacies in Bern (Rathaus Apotheke Bern; 2002-2003), Basel (Sunstore Apotheke Pratteln; 2005-2006), Affoltern am Albis (Amavita Apotheke, 2006-2010).

6.3 Publications

Original publications

Knabl J, **Witschi R**, Hösl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Möhler H, Zeilhofer HU. (2008) Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes. *Nature* 451:330-334.

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Zeilhofer HU, **Witschi R**, Hösl K. (2009) Subtype-selective $GABA_A$ receptor mimetics--novel antihyperalgesic agents? *J Mol Med* 87:465-769.

Book chapters

Zeilhofer HU, **Witschi R**, Johansson T (2009) **Fast Inhibitory Transmission of Pain in the Spinal Cord**" in: Malcangio M (ed): Synaptic Plasticity in Pain. Springer, New York.

6.4 Poster Presentations, Talks and Awards

Poster Presentations (only first author poster presentations listed; chronologically)

"GABAergic Control of Pain Processing", ***ZNZ Symposium 2006***, Zürich, 20.10.2006

"Reversal of Neuropathic Pain through Specific Spinal $GABA_A$ Receptor Subtypes" and "Spinal Endocannabinoids Mediate Activity-Dependent Central Sensitization in Pain Pathways, Part II (Behavior)", ***ZNZ Symposium 2007***, Zürich, Switzerland, 14.09.2007

"Generation of a Hoxb8-Cre Mouse Line for Brain-Sparing Gene Deletion", ***Annual Meeting 2007 of the Swiss Society of Pharmacology & Toxicology,*** Zürich, Switzerland, 27.-28.09.2007

"Spinal Endocannabinoids Mediate Activity-Dependent Central Sensitization in Pain Pathways", ***Neuroscience Meeting***, San Diego, USA, 3.-7.11.2007

"Contribution of $GABA_A$ Receptors on Nociceptive Primary Afferent Terminals to Antihyperalgesia by Intrathecal Benzodiazepines", ***Zurich Pharmacology Posterday 2008***, UZH/ETH Zurich, Zürich, Switzerland, 27.5.2008

"Generation of a Hoxb8-Cre Mouse Line for Brain-Sparing Gene Deletion" and "Contribution of $GABA_A$ Receptors on Nociceptive Primary Afferent Terminals to Antihyperalgesia by intrathecal Benzodiazepines", ***ZNZ PhD Retreat***, Valens, Switzerland, 29.- 31.5.2008

"Contribution of $GABA_A$ Receptors on Nociceptive Primary Afferent Terminals to Antihyperalgesia by Intrathecal Benzodiazepines", ***FENS***, Geneva, Switzerland, 12.-16.7.2008

"Spinal Endocannabinoids Mediate Activity-Dependent Central Sensitization in Pain Pathways" and "Contribution of $GABA_A$ Receptors on Nociceptive Primary Afferent Terminals to Antihyperalgesia by Intrathecal Benzodiazepines", ***World Congress on Pain*** (IASP organisation), Glasgow, UK, 17.-22.08.2008

"Contribution of $GABA_A$ Receptors on Nociceptive Primary Afferent Terminals to Antihyperalgesia by Intrathecal Benzodiazepines", ***ZNZ Symposium 2008***, Zürich, Switzerland, 12.09.2008

"Contribution of $GABA_A$ Receptors on Nociceptive Primary Afferent Terminals to Antihyperalgesia by Intrathecal Benzodiazepines", ***Zurich Pharmacology Posterday 2009***, UZH/ETH Zurich, Zürich, Switzerland, 10.07.2009

Talks (outside)

"$GABA_A$ Receptor Subunit-Specific Analgesia", ***European Winter Conference on Brain Research*** (EWBCR), Les Menuires, France, 7.-14.03.2009

Awards

1st prize ***"Deutscher Förderpreis für Schmerzforschung 2008"*** in the category „basic research" for the publication "Reversal of pathological pain through specific spinal $GABA_A$ receptor subtypes" by ***DGSS*** (Deutsche Gesellschaft zum Studium des Schmerzes), sponsored by Grünenthal GmbH. Berlin, Germany, 2008.

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Illustration of René Descartes' theory of pain, in which a painful sensation travels along a specific "pain pathway" up the spinal cord to be perceived in the brain,1664.